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Unique patterns of BTK resistance: two independently arising resistance clones in response to covalent BTK inhibitor therapy in CLL/SLL

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Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is a mature B-cell neoplasm composed of small atypical lymphoid cells that often coexpress CD5 and CD23 and are characterized by scant cytoplasm, clumped nuclear chromatin, and indistinct nucleoli. CLL/SLL can involve the peripheral blood, bone marrow, and various lymphoid tissues such as the lymph nodes, tonsils, and spleen, and it may occasionally present in extranodal locations as well.¹ Within involved lymph nodes, pale-staining proliferation centers consisting of prolymphocytes or paraimmunoblasts are a characteristic finding in CLL/SLL.

CLL/SLL makes up 25 to 30 percent of total leukemias in the United States, predominantly affecting older adults with an average age of 70 and demonstrating a slightly higher incidence in male patients.² The clinical course of CLL/SLL is heterogeneous, ranging from indolent to aggressive disease. Cytogenetic and molecular features play a crucial role in the initial prognostication of CLL/SLL, and it is important to consider that certain molecular markers can evolve over the course of the disease, potentially leading to drug resistance and disease progression. Covalent Bruton tyrosine kinase (BTK) inhibitors, such as ibrutinib, have become a cornerstone of CLL/SLL therapy, used both as first-line treatment and for managing disease progression. However, mutations in the *BTK* gene can result in acquired resistance to these therapies, making it essential to assess a patient's *BTK* mutation status for effective treatment planning.

Here, we present two male patients with CLL/SLL who experienced disease progression and were found to have a similar mutational profile, with each patient having the rare occurrence of two independently arising resistance clones, both of which resulted in the *BTK* p.C481S ibrutinib resistance mutation.

Case No. 1. A 75-year-old male smoker presented in 2019 with bilateral cervical lymphadenopathy. CBC showed leukocytosis with absolute lymphocytosis. CT revealed exten-

sive lymphadenopathy above and below the diaphragm, including a 7.4-cm retroperitoneal nodal conglomerate and mild splenomegaly. An excisional lymph node biopsy confirmed CD5-positive B-cell non-Hodgkin lymphoma, consistent with CLL/SLL. Flow cytometry showed an abnormal B-cell population with bright CD5 and CD200, intermediate CD22, five percent CD38, dim kappa restriction, decreased CD20, and negative CD3, CD10, and CD56. Subsequent bone marrow biopsy confirmed CLL with del(17p) and trisomy 12 on FISH and a complex karyotype. Ibrutinib therapy induced remission but caused atrial fibrillation, prompting a switch to a well-tolerated acalabrutinib in 2021.

The patient was recently hospitalized for increased fatigue and abdominal pain, with laboratory studies revealing elevated bilirubin levels (total: 5.2 mg/dL, direct: 2.7 mg/dL), AST (184 U/L), and ALT (282 U/L), while abdominal MRI showed new lymphadenopathy (up to 2 cm) and hepatomegaly. Peripheral blood flow cytometry identified an abnormal B-cell population (33.8 percent of leukocytes) expressing CD5, consistent with persistent disease. Subsequent lymphoid gene panel testing revealed clinically significant variants, including *NOTCH1* (p.P2514Rfs*4, NM_017617.3:c.7541_7542del, VAF 16 percent), two distinct *BTK* variants (p.C481S, NM_000061.3:c.1441T>A, VAF 11 percent, and p.C481S,

NM_000061.3:c.1442G>C, VAF 10 percent), and *TP53* (p.R110P, NM_000546.5:c.329G>C, VAF three percent). The C481S mutations, associated with resistance to covalent BTK inhibitors like ibrutinib and acalabrutinib, necessitated a change in therapy to obinutuzumab and venetoclax.

Case No. 2. A 65-year-old male patient known to have chronic lymphocytic leukemia characterized by unmutated *IGHV* and a complex karyotype was diagnosed in 2014 and managed initially with a watch-and-wait strategy for four years. In 2018, due to rising WBC counts and symp-

tomatic lymphadenopathy, the patient was enrolled in protocol 9905 and randomized to full-dose zanubrutinib (160 mg BID). Treatment was well tolerated with minimal toxicity, and CBC showed no lymphocytosis (ALC <4000) or cytopenias, indicating an excellent response.

Six years later, laboratory tests showed declining platelets (141k), neutropenia (ANC 1.36), and rising ALC (8.46). The patient remained asymptomatic but had palpable lymphadenopathy, including an approximately 1-cm right cervical and an approximately 2-cm left axillary

node, suggesting disease progression. Molecular testing for *BTK* single-gene analysis revealed two variants: p.C481S, NM_000061.3:c.1441T>A, VAF 36 percent, and p.C481S, NM_000061.3:c.1442G>C, VAF 12 percent. No clinically significant variants were identified in *PLCG2*. With evidence of progression and based on molecular results, zanubrutinib was stopped, and the patient started on obinutuzumab and venetoclax.

Both cases highlight the evolution of CLL/SLL, with *BTK* C481S mutations driving resistance and necessitating alternative therapeutic strategies, such as obinutuzumab-venetoclax-based regimens, to manage relapsed/refractory CLL. Fortunately, both patients are currently doing well clinically and remain on obinutuzumab and venetoclax therapy. For the patient in case No. 1, the current line of therapy has resulted in resolution of his lymphadenopathy and a normal white blood cell count. In the event of disease progression, next-line therapy options would include pirtobrutinib, CAR T-cell therapy, or clinical trials. The patient in case No. 2 was able to decrease the frequency of his surveillance visits from monthly to every three months given his stable clinical status.

Methods. Both samples were evaluated using a multigene next-generation sequencing panel designed to detect mutations associated with hematologic disorders. Purified genomic DNA was enzymatically fragmented, and targeted sequences were isolated by hybrid capture probes and sequenced on an Illumina NGS platform. The nucleic acid extraction, amplification, sequencing, and data interpretation are performed in the UW molecular hematopathology laboratory. A custom bioinformatics pipeline developed by the UW NGS analytics laboratory was used for data analysis as previously described.³

The samples from these two patients were processed on different days and were on different runs of

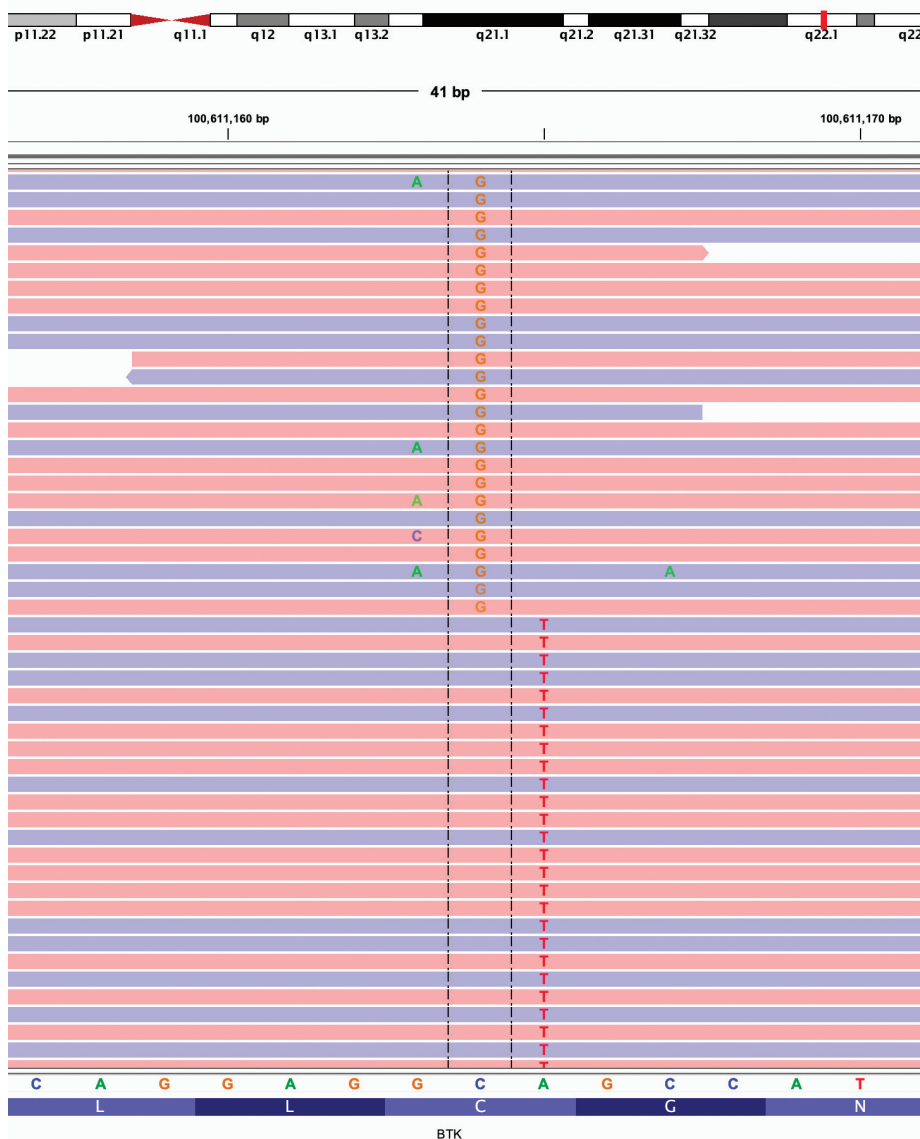


Fig. 1. Sequencing visualization using Integrative Genomics Viewer for patient No. 1 demonstrating the presence of two mutations affecting the C481 residue within the *BTK* gene. The two mutations are not present within the same reads as one another, meaning they are not in-cis and do not affect the same *BTK* allele.

this NGS assay, minimizing concerns about sample contamination.

Discussion. Some CLL/SLL patients experience indolent disease and may not require treatment for many years, while others may face rapid progression of disease. Clinical features, such as the degree of lymphocytosis and the presence of hepatomegaly or splenomegaly, anemia, and thrombocytopenia, as well as molecular and cytogenetic features—including evaluation of del(11q), del(13q), del(17p), trisomy 12, *TP53* mutation analysis, and *IGHV* gene mutation status—play a critical role in staging and prognostication for patients with newly diagnosed CLL/SLL.¹

Prior to the advent of BTK inhibitors, the mainstay of treatment for CLL/SLL was typically a combination of chemotherapy and immunotherapy. BTK is a kinase involved in B-cell receptor signaling and is up-regulated in CLL/SLL cells compared with normal B cells. Targeted inhibition of BTK blocks B-cell receptor signaling via covalent binding of the *BTK* C481 residue, thus diminishing proliferation signals in the neoplastic cells.^{4,5} This blockade has been shown to translate clinically into improved survival outcomes for CLL/SLL patients treated with BTK inhibitors compared with those receiving prior standard therapies.⁶ The approval of the BTK inhibitor ibrutinib in 2014 for patients who had received at least one prior therapy, and in 2016 for untreated CLL, has dramatically transformed the therapeutic landscape for CLL/SLL.

BTK inhibitor therapy has been shown to lead to durable remission in many CLL/SLL patients. In fact, long follow-up from the pivotal RESONATE-2 study demonstrated that the estimated five-year progression-free survival rate was 70 percent for patients treated with first-line ibrutinib and 12 percent for those treated with chlorambucil.⁷ Follow-up at eight years demonstrated continued benefit with ibrutinib therapy, with a

PFS rate of 59 percent for ibrutinib and nine percent for chlorambucil.⁸ Notably, the improvement in PFS seen with first-line ibrutinib as compared with chlorambucil was consistent across CLL patient groups, including those with high-risk genetic features, such as del(11q), *TP53* mutation, and/or unmutated *IGHV*, and those with advanced-stage disease.⁸

Although many patients are able to achieve long-term remission with BTK inhibitor therapy, a subset of patients will still develop progressive disease after an initial response to therapy due to acquired resistance to ibrutinib (or other BTK inhibitors). Resistance to targeted BTK inhibition therapy has been attributed to the development of resistance mutations in certain key genes, most commonly involving *BTK* and/or *PLCG2*. Within the *BTK* gene, the most common resistance mutations are p. C481S (substitution of cysteine with serine) and p.C481R (substitution of cysteine with arginine). These alterations interfere with the ability of ibrutinib to covalently bind to and diminish BTK activity.⁹ Similarly, zanubrutinib and acalabrutinib also rely on covalent binding to the C481 residue to exert their effects. As a result, the common p.C481S and p.C481R mutations also confer resistance to these second-generation BTK inhibitors.

Pirtobrutinib is a third-generation BTK inhibitor that does not rely on covalent binding of the C481 residue; rather, it noncovalently/reversibly inhibits BTK within the adenosine triphosphate-binding site. Pirtobrutinib was found to be a promising treatment option for patients with pretreated CLL, including those with a *BTK* C481 resistance mutation.^{10,11} Patients with progressive disease on pirtobrutinib therapy have been found to have resistance mutations other than the canonical C481 mutations, such as T474 codon mutations and L528W.¹² *PLCG2* is downstream of *BTK* in the B-cell receptor signaling pathway, and resistance mutations

occurring in *PLCG2* tend to be gain-of-function alterations that allow for continued signaling within the B-cell receptor pathway despite BTK blockade.^{9,15} Different BTK inhibitors exhibit varying levels of specificity against resistance mutations, influencing their efficacy. For example, ibrutinib demonstrates the widest range of coverage against non-C481 mutations, while acalabrutinib is potent against the L528W mutation but less effective against T474I, and zanubrutinib is inactive against L528W but modestly effective against T474I.¹³ These findings underscore the importance of *BTK* gene sequencing during a patient's treatment course, particularly when disease progression is suspected, with the ability to detect not only resistance mutations at the common C481 site but also those at other locations within the gene.¹⁴

Regarding the two patients described in this report, upon clinical disease progression, both were found to have the common *BTK* p.C481S mutation, which causes resistance to covalent BTK inhibitors such as ibrutinib, acalabrutinib, and zanubrutinib. Notably, each patient had two separate mutations, both of which resulted in the *BTK* p.C481S mutation. The first was a T>A substitution at nucleotide 1441 (NM_000061.3:c.1441T>A), and the other a G>C substitution at nucleotide 1442 (NM_000061.3:c.1442G>C). Based on review of the sequencing data in Integrative Genomics Viewer (IGV), the two *BTK* variants were never located on the same read with one another ("not in phase"), meaning they did not occur in the same physical *BTK* allele. The *BTK* gene is located on the X chromosome; therefore, if the two patients had been female with two X chromosomes, observing such a pattern in IGV could mean that the two variants were within the same cell but in trans (on different alleles), or involving two different cell clones. Since both of our patients were male, with only one X chromosome each, the most likely

explanation for the variants not being in phase is that they are present within two different independently arising clonal populations.

The presence of two independently arising *BTK* p.C481S mutations is a pattern of resistance mutations that has not been commonly reported. There have been reports of relapsing CLL/SLL patients harboring more than one *BTK* mutation; however, the mutations have been different from one another (for example, *BTK* p.C481S co-occurring with *BTK* p.T474I, p.C481R, or p.C481Y).¹⁶⁻¹⁹ The clinical impact of two separate clonal populations, each with a *BTK* p.C481S mutation, as observed in the two patients described in this report, compared with the impact of p.C481S in conjunction with a different *BTK* mutation, is unclear. However, it can be assumed that the presence of any two *BTK* resistance mutations likely contributes to increased drug resistance.

In summary, the patients described in this report show a unique pattern of *BTK* resistance mutations, with two different, independently arising clonal populations. Further studies are needed to better understand the clinical significance of this pattern of *BTK* mutations, and their role in drug resistance, which may help guide more effective treatment strategies for CLL/SLL patients. □

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Test yourself

Here are three questions taken from the case report. Answers are online now at www.amp.org/casereports and will be published next month in CAP TODAY.

1. What was the name of the first targeted *BTK* inhibitor used in the treatment of chronic lymphocytic leukemia/small lymphocytic lymphoma?
 - a. Zanabrutinib
 - b. Ibrutinib
 - c. Acalabrutinib
 - d. Orelabrutinib
2. Resistance mutations in CLL/SLL are most commonly seen in which two genes?
 - a. *BLNK* and *SYK*
 - b. *BTK* and *PLCG2*
 - c. *CD19* and *BTK*
 - d. *BLK* and *LYN*
3. What is the genetic mechanism behind *BTK* p.C481S mutations?
 - a. Loss of function leading to apoptosis
 - b. Gain of function causing continuous B-cell receptor signaling
 - c. Prevents covalent binding of *BTK* inhibitors
 - d. Enhances *TP53* gene activity