

## ORIGINAL ARTICLE

# Resistance Mechanisms for the Bruton's Tyrosine Kinase Inhibitor Ibrutinib

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## ABSTRACT

**BACKGROUND**

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Ibrutinib is an irreversible inhibitor of Bruton's tyrosine kinase (BTK) and is effective in chronic lymphocytic leukemia (CLL). Resistance to irreversible kinase inhibitors and resistance associated with BTK inhibition have not been characterized. Although only a small proportion of patients have had a relapse during ibrutinib therapy, an understanding of resistance mechanisms is important. We evaluated patients with relapsed disease to identify mutations that may mediate ibrutinib resistance.

**METHODS**

We performed whole-exome sequencing at baseline and the time of relapse on samples from six patients with acquired resistance to ibrutinib therapy. We then performed functional analysis of identified mutations. In addition, we performed Ion Torrent sequencing for identified resistance mutations on samples from nine patients with prolonged lymphocytosis.

**RESULTS**

We identified a cysteine-to-serine mutation in *BTK* at the binding site of ibrutinib in five patients and identified three distinct mutations in *PLCγ2* in two patients. Functional analysis showed that the C481S mutation of *BTK* results in a protein that is only reversibly inhibited by ibrutinib. The R665W and L845F mutations in *PLCγ2* are both potentially gain-of-function mutations that lead to autonomous B-cell-receptor activity. These mutations were not found in any of the patients with prolonged lymphocytosis who were taking ibrutinib.

**CONCLUSIONS**

Resistance to the irreversible BTK inhibitor ibrutinib often involves mutation of a cysteine residue where ibrutinib binding occurs. This finding, combined with two additional mutations in *PLCγ2* that are immediately downstream of BTK, underscores the importance of the B-cell–receptor pathway in the mechanism of action of ibrutinib in CLL. (Funded by the National Cancer Institute and others.)

THE DEVELOPMENT OF B-CELL-RECEPTOR antagonists has been a therapeutic advance in chronic lymphocytic leukemia (CLL). Although B-cell-receptor ligation in normal cells induces proliferation, apoptosis, or anergy,<sup>1</sup> pathway dysregulation in CLL results in the propagation of proliferative and prosurvival signals.<sup>2,3</sup> Several agents targeting the B-cell-receptor pathway are in development, including the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib. Although *BTK* is not recurrently mutated in CLL,<sup>4,5</sup> it is up-regulated at the transcript level and is constitutively active.<sup>6,7</sup> Ibrutinib irreversibly binds BTK at the C481 residue, rendering it kinase-inactive, inducing modest CLL-cell apoptosis, and abolishing proliferation and B-cell-receptor signaling in vitro.<sup>6,8</sup> Ibrutinib has been shown to have clinically significant activity in patients with relapsed CLL, with 71% of patients having an objective complete or partial response and an additional 15 to 20% of patients having a partial response with persistent lymphocytosis. At 26 months, the estimated progression-free survival rate among patients treated with ibrutinib is 75%.<sup>9</sup> Few patients have had a relapse, but as more patients are treated with ibrutinib, it becomes increasingly important to identify mechanisms of acquired resistance in order to offer effective salvage therapies. In addition, determining whether persistent lymphocytosis has similar resistant features could affect treatment choices for patients with prolonged lymphocytosis during ibrutinib therapy.

The model for kinase inhibition in hematologic cancers is the BCR-ABL inhibitor imatinib, which transformed therapy for chronic myeloid leukemia.<sup>10</sup> The most common mechanisms of acquired resistance to imatinib are point mutations in the kinase domain of ABL. Although the T315I mutation is the most common,<sup>11,12</sup> more than 100 resistance mutations have been identified that prevent imatinib binding through binding-site alteration or destabilization of the inactive conformation of ABL.<sup>13</sup> Because *BTK* has not been identified as a mutated gene in CLL, whereas BCR-ABL has been shown to be a mutational hot spot,<sup>14</sup> it is uncertain whether the type of resistance seen with imatinib will be relevant to CLL. In addition, ibrutinib is an irreversible inhibitor of BTK through its ability to bind to the C481 site, distinguishing it from imatinib and other reversible kinase inhibitors that have been studied in cancer to date. How cancer cells,

including CLL cells, develop resistance to ibrutinib or other irreversible inhibitors is still unknown. The development of mutations in genes that reactivate downstream B-cell-receptor signaling or other pathways is certainly possible, because clonal evolution is common in previously treated CLL.<sup>15</sup> We evaluated patients who had CLL and acquired resistance to ibrutinib for mutations that may mediate resistance.

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## METHODS

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### DNA SEQUENCING

We obtained blood samples from patients enrolled in institutional review board-approved trials of ibrutinib. One of the patients (Patient 1) is described extensively in the *Journal* by Furman et al.<sup>16</sup> Tumor DNA was isolated from blood mononuclear cells with the use of the AllPrep DNA/RNA Mini Kit (Qiagen). Sample preparation and whole-exome sequencing with the use of Agilent SureSelect Human All Exon V4 and Illumina HiSeq 2000 technology were performed by Expression Analysis.

### DATA-ANALYSIS WORKFLOW

The exome-sequencing analysis pipeline is shown in Figure 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org. Sequencing reads were aligned to the human reference genome (1000 Genomes Project human assembly GRCh37) with the use of Burrows-Wheeler Aligner, version 0.7.5.<sup>17</sup> After potential polymerase-chain-reaction or optical duplicates had been marked with the use of Picard, version 1.94 (<http://picard.sourceforge.net>), local realignment around indels was performed by means of the Genome Analysis Toolkit (GATK), version 2.8.1,<sup>18</sup> and relapse-specific single point mutations and indels were detected with the use of MuTect, version 1.1.4,<sup>19</sup> and GATK Somatic Indel Detector, respectively. Variants previously reported in the dbSNP database, build 137, were filtered out, and the remaining variants were annotated and their potential mutational effects predicted with the use of SnpEff, version 3.4.<sup>20</sup> Finally, newly acquired, relapse-specific, nonsynonymous mutations with high-quality reads were verified by means of Sanger or Ion Torrent sequencing.

### ION TORRENT ANALYSIS

DNA was extracted from cryopreserved cells with the use of the QIAamp DNA Mini Kit (Qiagen).

*BTK* and *PLCγ2* were analyzed with the use of the Ion Torrent platform from Life Technologies. Details of sample preparation, library generation, and sequence analysis are provided in the Supplementary Appendix.

#### DNA CONSTRUCTS AND CELL CULTURE

The PiggyBac Transposon Vector System (System Biosciences) was used to generate DNA constructs of *BTK* with both green-fluorescent-protein and puromycin selection markers, and protein expression was controlled with the use of chicken  $\beta$ -actin promoter. See the Supplementary Appendix for details.

#### IN VITRO KINASE ASSAY

Full-length His6- and Strep-tagged *BTK* and *BTK* C481S were expressed in mammalian HEK-EBNA cells and purified by means of affinity chromatography. Purified proteins (100 ng per milliliter) were used in a LANCE Ultra kinase assay (Perkin-Elmer), with 100 nM ULIGHT-poly GT (Perkin-Elmer) as a substrate and 200  $\mu$ M ATP. Compounds were preincubated at room temperature with enzyme before the kinase reaction. After 45 minutes of kinase reaction, excess EDTA was added, and a europium-labeled anti-poly-GT antibody was incubated before signal measurement in a dual-laser EnVision plate reader (PerkinElmer), set at an excitation wavelength of 320 nm and an emission wavelength of 615 to 665 nm.

#### FLOW-CYTOMETRIC AND IMMUNOBLOT ASSAYS

HEK 293T cells were transiently transfected with the indicated expression constructs, treated with ibrutinib for 1 hour, and fixed with paraformaldehyde or washed into fresh media and then fixed. Cells were permeabilized, stained, and analyzed on a BD FACSCanto II analyzer (BD Biosciences).

For immunoblot assays, whole-cell lysates were prepared and equivalent amounts of protein were separated on polyacrylamide gels and transferred onto nitrocellulose membranes. After antibody incubation, proteins were detected with the use of a chemiluminescent substrate (SuperSignal, Pierce). Details on the antibodies used are provided in the Supplementary Appendix.

#### STATISTICAL ANALYSIS

The statistical methods for individual experiments are detailed in the Supplementary Appendix.

## RESULTS

### MUTATIONS IN *BTK* AND *PLCγ2* REVEALED BY WHOLE-EXOME SEQUENCING

Peripheral-blood samples were available at baseline and at the time of relapse from six patients with acquired resistance to ibrutinib manifested by progressive CLL. Whole-exome sequencing was performed on each sample. Clinical characteristics and new mutations identified at the time of relapse in the patients with matched samples are shown in Table 1, and Table 1 in the Supplementary Appendix. Alignment statistics are shown in Table 2 in the Supplementary Appendix. Copy-number analysis was performed to ensure that identified variants were not a result of potential copy-number alterations (Table 3 and Fig. 2 in the Supplementary Appendix). All patients had high-risk cytogenetic features, such as del(11q22.3), del(17p13.1), or a complex karyotype.

In Patients 1 through 5, sequencing of the relapse sample revealed a cysteine-to-serine mutation in *BTK* at position 481 (C481S) (Fig. 3A in the Supplementary Appendix). In Patient 6, the relapse sample had an arginine-to-tryptophan mutation in *PLCγ2* at position 665 (R665W) (Fig. 3B in the Supplementary Appendix). One patient with a low-frequency C481S mutation in *BTK* also had three distinct *PLCγ2* mutations: the R665W mutation, a leucine-to-phenylalanine mutation at position 845 (L845F) (Fig. 3C in the Supplementary Appendix), and a serine-to-tyrosine mutation at position 707 that was previously reported as an activating mutation in a dominantly inherited inflammatory disease.<sup>21</sup> In this patient, *BTK* C481S and *PLCγ2* L845F were identified by means of whole-exome sequencing, and the other mutations were identified by means of Ion Torrent sequencing. To verify these clones, Ion Torrent sequencing was performed again on samples obtained 1 month after relapse, and all previously identified mutations were still present (Table 4 in the Supplementary Appendix). Mutations identified by whole-exome sequencing were confirmed by Sanger sequencing, Ion Torrent deep sequencing, or both.

At baseline, no patient had evidence of mutations in either *BTK* or *PLCγ2* on the basis of whole-exome sequencing. In Patients 3, 5, and 6, Ion Torrent sequencing was performed, and no mutation was identified in more than 0.2% of reads (Table 5 in the Supplementary Appendix).

**Table 1. Characteristics of Six Patients with Resistance to Ibrutinib.**

Patient No.	Age yr	Prior Therapies no.	Baseline Cytogenetic Features*	Study Treatment and Daily Dose†	Duration of Ibrutinib Treatment days	Best Response	Time to First Response days	Identified Mutations of Interest‡
1	59	5	del(17p13.1), trisomy 12	Ibrutinib, 560 mg	621	Partial	70	C481S mutation in BTK
2	59	3	del(11q22.3)	Bendamustine–rituximab for 6 cycles; ibrutinib, 420 mg	388	Complete	70	C481S mutation in BTK
3	51	2	complex karyotype	Ofatumumab for 24 wk; ibrutinib, 420 mg	674	Complete	85	C481S mutation in BTK
4	69	9	del(17p13.1), complex karyotype	Ibrutinib, 840 mg	868	Partial	133	C481S mutation in BTK
5	61	4	del(17p13.1), complex karyotype	Ofatumumab for 24 wk; ibrutinib, 420 mg	505	Partial	85	L845F, R665W, and S707Y mutations in PLC $\gamma$ 2 and C481S mutation in BTK
6	75	2	del(17p13.1), complex karyotype	Ibrutinib, 420 mg	673	Partial	159	R665W mutation in PLC $\gamma$ 2

\* We used fluorescence in situ hybridization to detect del(17p13.1), del(11q22.3), centromere 12, and del(13q14.3) and metaphase analysis of stimulated G-banded cells to determine complexity.

† Doses are given for ibrutinib only.

‡ All functional mutations that were detected only at the time of relapse are listed in Table 1 in the Supplementary Appendix.

At relapse, none of the patients had mutations in other kinases containing a cysteine residue homologous to C481 in *BTK* that are irreversibly inhibited by ibrutinib (data on *ITK*, *BMX*, *TEC*, *EGFR*, *JAK3*, *HER2*, *HER4*, and *BLK* not shown).<sup>7,22</sup> No other recurrent mutation was noted in any of these six patients at the time of relapse.

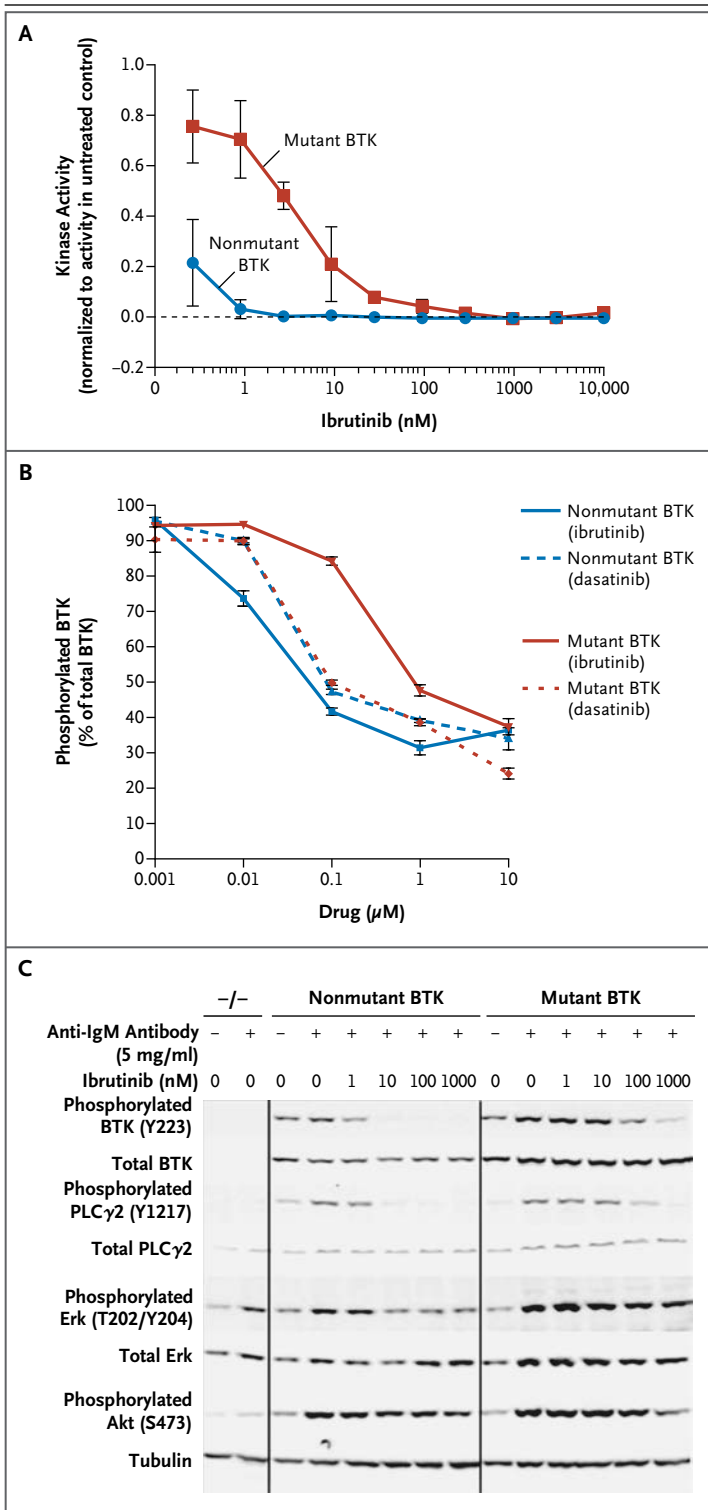
#### REVERSIBLE INHIBITION OF MUTANT BTK BY IBRUTINIB IN VITRO AND IN PATIENTS

We performed functional characterization of non-mutant BTK and BTK with the C481S mutation. The intrinsic affinity of ibrutinib for nonmutant BTK was significantly higher than for mutant BTK (dissociation constant, 0.2 nM vs. 10.4 nM) (Table 6 in the Supplementary Appendix). Although both nonmutant and mutant BTK were inhibited by ibrutinib, the half-maximal effective concentration for BTK with the C481S mutation was significantly higher than for nonmutant BTK (Fig. 1A). In a cellular model, ibrutinib was significantly less effective at blocking BTK autophosphorylation (Fig. 1B) and downstream signaling (as indicated by phosphorylated ERK levels) in cells with mutant BTK than in cells with nonmutant BTK (Fig. 4A in the Supplementary Appendix), whereas dasatinib, a reversible inhibitor of BTK,<sup>23</sup> inhibited nonmutant and mutant BTK

with similar efficacy. Ibrutinib washout experiments showed that the C481S mutation allows reversible, but not irreversible, inhibition of BTK by ibrutinib, which was confirmed by kinase-binding assays (Fig. 4B and Table 6 in the Supplementary Appendix).

Next, we compared the function of nonmutant BTK and BTK with the C481S mutation by stably transfecting constructs into DT40 (BTK<sup>-/-</sup>) chicken B cells. After activation of the B-cell receptor, signaling downstream of BTK was inhibited by ibrutinib, as shown by decreased downstream signaling (phosphorylation of PLC $\gamma$ 2, ERK, and AKT) and calcium flux; this inhibition was diminished in mutant BTK as compared with nonmutant BTK (Fig. 1C, and Fig. 5 in the Supplementary Appendix). These data clearly show that the C481S mutation in *BTK* confers relative resistance to ibrutinib by preventing irreversible binding, providing confirmation that this is a functionally relevant resistance mutation.

We compared BTK phosphorylation on exposure to ibrutinib in cells obtained at baseline and at the time of relapse from one of the patients in whom a *BTK* C481S mutation developed. At baseline, BTK phosphorylation was completely abrogated by ibrutinib with either washout or continuous exposure to ibrutinib. At the time of



**Figure 1. Functional Characterization of Bruton's Tyrosine Kinase (BTK) with the C481S Mutation.**

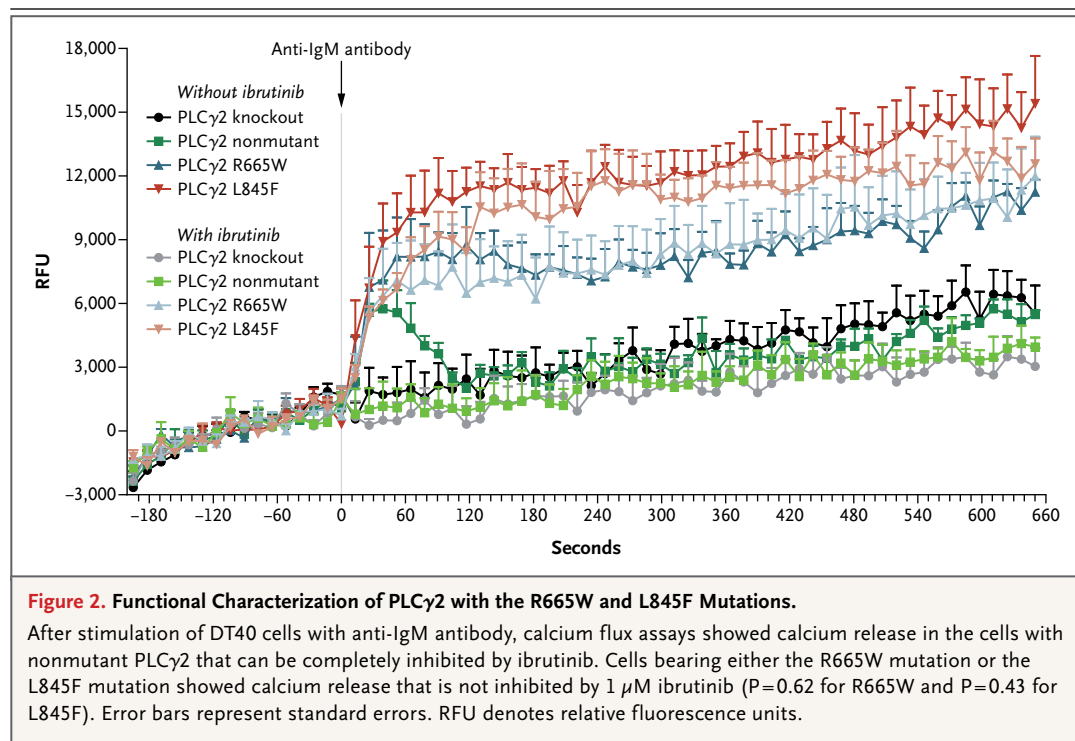
An assay of recombinant nonmutant BTK versus BTK with the C481S mutation (mutant BTK) showed that the mutant form had enhanced kinase activity that could be inhibited by ibrutinib, though at a much higher half-maximal effective concentration than with nonmutant BTK (Panel A). The data shown reflect two independent experiments with triplicate samples for each treatment. After transfection of mutant or nonmutant BTK into HEK 293T cells, administration of ibrutinib did not inhibit B-cell–receptor signaling in cells with mutant BTK to the same degree as in cells with nonmutant BTK. BTK autophosphorylation at tyrosine 223 (Y223) (Panel B) was significantly more inhibited in cells with nonmutant BTK than in cells with mutant BTK, at 0.01 μM, 0.1 μM, and 1 μM of ibrutinib (P<0.001), and the difference in inhibition between nonmutant BTK and mutant BTK was significantly greater with ibrutinib than with dasatinib (P<0.001). The data shown in Panels A and B are normalized. I bars in both panels indicate standard errors. After transfection of nonmutant or mutant BTK into BTK<sup>-/-</sup> DT40 cells, B-cell–receptor signaling was inhibited by ibrutinib in cells with nonmutant BTK to a greater degree than in cells with mutant BTK (Panel C). The data shown in Panels B and C reflect at least three independent experiments.

tant reversibly in patients (Fig. 6 in the Supplementary Appendix).

**IDENTIFIED MUTATIONS IN PLCγ2 AS RESISTANCE MECHANISMS IN VITRO AND IN PATIENTS**

It has been shown that the S707Y mutation in PLCγ2 has a gain-of-function effect owing to disruption of an autoinhibitory SH2 domain.<sup>21</sup> We therefore chose to focus on functional characterization of the R665W and L845F mutations. We stably transfected nonmutated PLCγ2, PLCγ2 with the L845F mutation, or PLCγ2 with the R665W mutation into HEK 293T cells and DT40 cells, which lack endogenous PLCγ2 expression (Fig. 7A in the Supplementary Appendix). We examined calcium flux in DT40 cells after stimulation with anti-IgM antibody in the presence of nonmutant or mutant PLCγ2. In contrast to BTK with the C481S mutation, for which calcium flux was very modestly inhibited by ibrutinib, PLCγ2 with either the R665W mutation or the L845F mutation showed enhanced IgM antibody–mediated calcium flux that was not inhibited by ibrutinib (Fig. 2). Thus, these mutations allow for B-cell receptor–mediated signaling that is independent of BTK.

relapse, however, ibrutinib inhibited BTK phosphorylation only with continuous exposure, a finding that shows that the drug binds the mu-



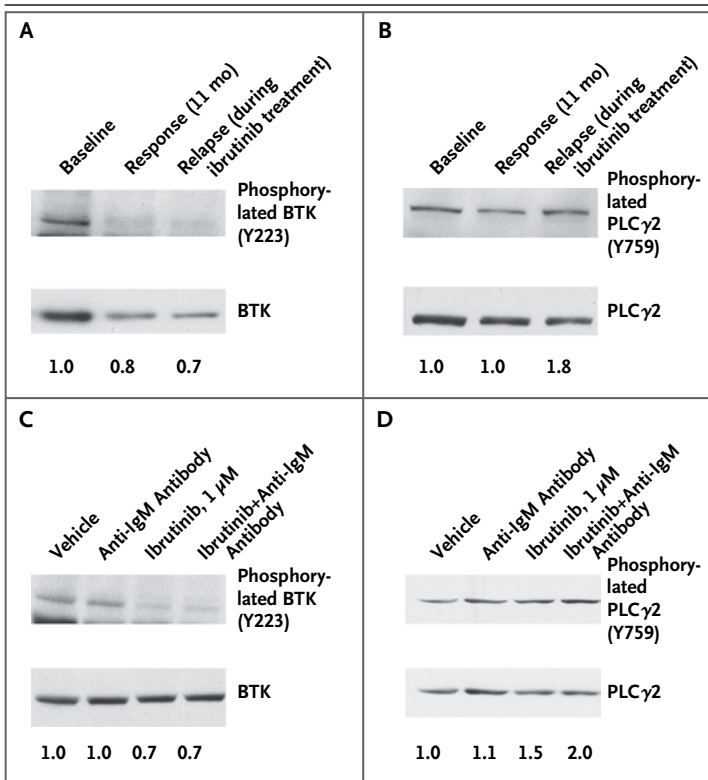
Similarly, after stimulation with anti-IgM antibody, cells with either the R665W mutation or the L845F mutation in PLCγ2 showed less inhibition in the presence of ibrutinib than nonmutant cells, as measured by phosphorylation of ERK and AKT (Fig. 7B in the Supplementary Appendix). These data show that the R665W and L845F mutations in PLCγ2 are potentially gain-of-function mutations in the presence of B-cell-receptor stimulation and could be relevant as mutations conferring resistance to ibrutinib in patients.

Finally, we examined CLL cells obtained at baseline and at the time of relapse from Patients 5 and 6. In Patient 6, the decrease in BTK phosphorylation at the time of the relapse was similar to the decrease at 11 months of therapy, when the patient was having a response to the drug (Fig. 3A), whereas PLCγ2 showed enhanced phosphorylation at the time of relapse (Fig. 3B). After ibrutinib was discontinued, ibrutinib still inhibited BTK phosphorylation (Fig. 3C) but not PLCγ2 phosphorylation (Fig. 3D). In Patient 5, who had mutations in both *BTK* and *PLCγ2*, in vitro ibrutinib did not inhibit either BTK or PLCγ2 phosphorylation (Fig. 8 in the Supplementary Appendix). These data suggest that the

gain-of-function phenotype seen in vitro is also relevant in patients.

#### ABSENCE OF *BTK* OR *PLCγ2* MUTATIONS IN PATIENTS WITH PROLONGED LYMPHOCYTOSIS DURING IBRUTINIB THERAPY

In patients treated with ibrutinib, a characteristic lymphocytosis develops as CLL cells are mobilized from lymph nodes and the spleen. Although lymphocytosis typically resolves within 8 months, a subgroup of patients have lymphocytosis that lasts longer than 12 months in the presence of a continued response to ibrutinib.<sup>24</sup> To determine whether such patients have new mutations in *BTK* or *PLCγ2* and may therefore be at risk for relapse, we sequenced these two genes using Ion Torrent technology in nine patients who had lymphocytosis for at least 12 months after the initiation of ibrutinib therapy. The sequencing depth for *BTK* at C481 and for *PLCγ2* at R665 and S707 was more than 700 reads, and the sequencing depth for *PLCγ2* at L845 was more than 100 reads. No patient had evidence of any mutation in *BTK* or *PLCγ2* (data not shown). These observations suggest that persistent lymphocytosis is not associated with known resistance mutations.



**Figure 3. Characterization of R665W PLCγ2 Mutation in a Patient.**

One patient who was found to have an R665W mutation in PLCγ2 had samples available for immunoblot analysis at baseline, during the period of response to ibrutinib, and at the time of relapse, before ibrutinib was discontinued. At baseline, BTK was phosphorylated, and this phosphorylation was inhibited by ibrutinib both during the period of response to the drug and at the time of relapse (Panel A). However, PLCγ2 showed evidence of sustained activation at the time of relapse through phosphorylation at tyrosine 759 (Y759) (Panel B), a finding that suggests that the R665W mutation is a gain-of-function mutation. At the time of relapse, after the drug had been discontinued, fresh cells were treated with vehicle, plate-immobilized anti-IgM antibody, 1 μM ibrutinib, or 1 μM ibrutinib plus anti-IgM antibody. BTK phosphorylation was inhibited by ibrutinib (Panel C), but PLCγ2 phosphorylation was not (Panel D); these findings show that the R665W mutation is not sensitive to ibrutinib in vitro. Densitometry values, shown below the lanes, reflect the ratio of phosphorylated protein to total protein in Panels B, C, and D; in Panel A, the values reflect the ratio of phosphorylated protein to loading control because total BTK protein expression can change over time during ibrutinib therapy.

allow B-cell receptor–mediated activation that is independent of BTK. Calcium-flux assays showed enhanced and sustained activation after anti-IgM antibody stimulation, which is probably very relevant in patients in whom CLL cells are exposed to chronic antigen stimulation. None of these mutations were detected before drug exposure. The clinical significance of the very low variant reads on Ion Torrent sequencing for the R665W mutation in PLCγ2 at baseline is unknown, and more patients will need to be evaluated to determine whether this signal is identifying patients at risk for relapse. We also found that patients with persistent lymphocytosis during ibrutinib treatment did not have evidence of these resistance mutations on deep sequencing. We have previously documented by immunoblot analysis<sup>24</sup> that BTK and PLCγ2 are inhibited in these patients, and these data suggest that patients with prolonged lymphocytosis during ibrutinib therapy do not have detectable small clones of resistant cells that would put them at high risk for relapse. However, we recognize that our inability to identify small numbers of mutant clones in the presence of large numbers of non-mutant clones may mean that such variants were present but were not identified.

Resistance to tyrosine kinase inhibitors through mutations that alter drug binding are seen with other targets and other cancers, including BCR-ABL in chronic myeloid leukemia,<sup>11</sup> EGFR<sup>25,26</sup> and ALK<sup>27</sup> in lung cancer, KIT in gastrointestinal stromal tumors,<sup>28</sup> and FLT3 in acute myeloid leukemia.<sup>29,30</sup> Unlike the mutations in this group, however, the C481S mutation in BTK is not a second mutation in a previously mutated gene, but rather a primary mutation in a gene that is not recurrently mutated in CLL, and it is mutated at the amino acid required for drug binding. This mutation is not known to cause the X-linked agammaglobulinemia phenotype<sup>31</sup> but instead results in retained catalytic activity. Our identification of a previously identified activating PLCγ2 mutation<sup>21</sup> and our functional characterization of the two additional mutations showed that the activation of B-cell–receptor signaling distal to BTK can also confer resistance. This pattern of a mutation localized to the irreversible binding site of ibrutinib and the immediate downstream kinase is not commonly seen with reversible kinase inhibitors and may be a result of continuous (rather than intermittent) pressure

## DISCUSSION

Acquired resistance to ibrutinib is due to at least in part to recurrent mutations in BTK and PLCγ2. Our functional studies suggest that the C481S mutation in BTK confers resistance to ibrutinib by preventing irreversible drug binding. The S707Y, R665W, and L845F mutations in PLCγ2 are all potentially gain-of-function mutations that

on the drug target. As other potent irreversible kinase inhibitors are developed for key signaling pathways in cancer, it will be of interest to determine whether this pattern of resistance is generalized. Studies of the stability of the mutated RNA and proteins are ongoing.

Although acquired resistance to ibrutinib has developed in only a small proportion of patients, these data suggest that patients with increased genomic instability, including those with del(17p13.1), del(11q22.3), or a complex karyotype, may be at risk for relapse. On the basis of available data, patients with del(17p13.1) or a complex karyotype seem to be the most rational choice for combination therapies designed to avoid the development of resistance.

Even though this series of patients had a uniform presence of mutations in *BTK*, *PLC $\gamma$ 2*, or both, it is clear that other mechanisms of resistance are also present in a subgroup of patients. In a recent preliminary study involving three patients with ibrutinib resistance, sequencing revealed the S707Y mutation in *PLC $\gamma$ 2* that we identified in Patient 5 in our study but did not reveal *BTK* mutations.<sup>32</sup> In patients without B-cell-receptor pathway mutations, resistance may be mediated through mutations in other coding genes providing alternative survival signals that are not inhibited by ibrutinib or through non-coding RNA, epigenetic activation or silencing, or

selective gene amplification. In addition, other mutations may act in combination with the *BTK* or *PLC $\gamma$ 2* mutations to drive resistance, such as the additional driver mutations outlined in Table 1 in the Supplementary Appendix. Work is ongoing to identify alternative mechanisms of resistance and also to determine the role, if any, of other identified mutations.

Although ibrutinib inhibits other similar kinases that contain C481, including *ITK* and *BLK*,<sup>7,22</sup> mutations have not been observed in these other targets, findings that suggest that *BTK* is its critical target. Knowledge of downstream mediators of resistance may lead to the development of rational combinations to prevent or treat resistant disease.

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