

B-cell receptor pathway mutations are infrequent in patients with chronic lymphocytic leukemia on continuous ibrutinib therapy [limit: ≤165 characters

including spaces, including spaces; no abbreviations; currently: 127 characters, including spaces]

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Running title: BCR pathway mutations during ibrutinib treatment [limit: ≤60 characters

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Translational Relevance Statement [limit: 120 – 150 words; currently: 149]

Limited data is available on the frequency of acquired mutations in Bruton's tyrosine kinase (*BTK*) or phospholipase C- γ 2 (*PLCG2*) genes in patients with chronic lymphocytic leukemia (CLL) treated with BTK inhibitors who have not yet experienced progressive disease (PD). Herein, we evaluated the frequency and time to detection of

BTK and *PLCG2* mutations that are associated with development of clinical PD in the peripheral blood of such patients with previously untreated or relapsed/refractory CLL across 5 clinical trials. The frequency of *BTK* mutations during ibrutinib treatment was lower, and time to development longer in previously untreated patients relative to patients with relapsed/refractory disease. Variability was observed in time from mutation to development of clinical PD, and mutations were not uniformly present in progressing patients. Hence, the detection of *BTK* or *PLCG2* mutations alone may not warrant changes in treatment in the absence of PD and should be further investigated.

Abstract: [limit 250-word structured abstract; subheadings included in count; current: 249]

Purpose: Acquired mutations in Bruton's tyrosine kinase (*BTK*) or phospholipase C- γ 2 (*PLCG2*) genes are associated with clinical progressive disease (PD) in patients with chronic lymphocytic leukemia (CLL) treated with BTK inhibitors. Data on mutation rates in patients without PD on ibrutinib treatment are limited.

Experimental Design: We evaluated frequency and time to detection of *BTK* and *PLCG2* mutations in peripheral blood samples from 388 patients with previously untreated (n=238) or relapsed/refractory (n=150) CLL across 5 clinical trials.

Results: With median follow-up of 35 months (range, 0-72) without PD at last sampling, mutations in *BTK* (3%), *PLCG2* (2%) or both genes (1%) were rare in previously untreated patients. With median follow-up of 35 months (range, 1-70) without PD at last sample, mutations in *BTK* (30%), *PLCG2* (7%), or both genes (5%) were more common in patients with relapsed/refractory CLL. Median time to first detection of *BTK* C481S mutation was not reached in previously untreated patients and was >5 years in patients with relapsed/refractory CLL. Among patients evaluable at PD, previously untreated patients (n=12) had lower rates than those with relapsed/refractory disease (n=45) of *BTK* (25% vs 49%) and *PLCG2* mutations (8% vs 13%). Time from first detection of *BTK* C481S mutation to PD was 11.3 months in 1 previously untreated patient and median 8.5 months (range, 0-35.7) among 23 patients with relapsed/refractory CLL.

Conclusions: This systematic investigation describes development of mutations over time in patients without PD and informs the potential clinical opportunity to optimize ongoing benefits for such patients.

Keywords: Ibrutinib; chronic lymphocytic leukemia; BTK mutations

Introduction

Ibrutinib, a once-daily Bruton's tyrosine kinase (BTK) inhibitor, is the only targeted therapy to date to demonstrate both a significant progression-free survival (PFS) and overall survival benefit in multiple randomized phase 3 studies in previously untreated and relapsed/refractory chronic lymphocytic leukemia (CLL)(1-6). While most patients achieve durable remissions, some eventually develop clinical resistance leading to progressive disease (PD), particularly in the relapsed/refractory setting.(7,8) In patients treated with ibrutinib across 4 clinical trials, most of whom had relapsed/refractory disease, the estimated cumulative incidence of CLL progression was 5%, 11%, and 19% at 2, 3, and 4 years, respectively (9). Previously untreated patients have lower rates of PD during ibrutinib treatment than relapsed/refractory patients: in patients with CLL treated with single-agent ibrutinib in RESONATE-2 and RESONATE, PFS rates at 5 years were 70% for previously untreated patients, compared with 60% in those with 1 or 2 prior therapies, and 33% in those with ≥ 3 prior therapies (10). Clinical risk factors associated with an increased risk of progression on ibrutinib therapy include del(17p)/*TP53* mutation, complex karyotype, and greater number of prior lines of therapy (7-9,11-13).

Acquired mutations in *BTK* or phospholipase C- $\gamma 2$ (*PLCG2*) genes have been frequently associated with clinical resistance to currently approved irreversible BTK inhibitors (7-9). These mutations have been observed in up to 80% of patients with CLL progression on ibrutinib or acalabrutinib (9,14,15). Several BTKis, including ibrutinib, bind irreversibly to the C481 residue in the BTK kinase domain, thereby preventing autophosphorylation of

the Y223 residue which is necessary for complete activation of the BTK kinase.(16-18) Most ibrutinib-resistant acquired *BTK* mutations result in an amino acid substitution at the C481 residue (most commonly C481S), which results in reversible ibrutinib binding at a lower affinity (7,9,14,19,20.) PLC γ 2 is the immediate downstream effector of BTK. Hotspot mutations in *PLCG2* are mostly gain-of-function mutations that result in proximal BCR activation of PLC γ 2 irrespective of BTK activity and thus continuous downstream BCR pathway signaling, independent of BTK activation (7,14).

To date, most information on *BTK* and *PLCG2* mutation rates comes from analyses conducted in patients who have experienced PD on ibrutinib therapy, and predominantly in patients with relapsed/refractory disease where mutations in *BTK* and *PLCG2* can be detected in advance of clinical evidence of progression (9,14). More limited data are available regarding such mutational rates in patients on ibrutinib treatment without PD (9). To better understand the extent of such mutations prior to PD, we assessed the frequency of *BTK* (focused on the most common C481 and T474 variants) and *PLCG2* mutations, as well as time to first detection of *BTK* C481S in previously untreated patients and those with relapsed/refractory disease who were continuing to respond to ibrutinib (ie, were free of PD) in clinical trials. Additional mutations were incidentally detected at the *BTK* L528 and R492 residues. Specifically, we hypothesized that mutations would be more common in patients with relapsed-refractory disease than in those previously untreated, in patients with 3 or more prior lines of treatment than in those with only 1 prior line of treatment, in those with del(17p) than in those without

del(17p), and in patients with relapsed-refractory disease and del(17p) than in previously untreated patients and del(17p).

Materials and methods

Patient samples

Peripheral blood samples were prospectively collected for separate analysis from 388 patients with CLL or small lymphocytic lymphoma (SLL) treated with ibrutinib in 5 clinical trials, including 3 trials in previously untreated patients and 2 trials in patients with relapsed/refractory disease. Detailed descriptions of these studies were previously reported (3,21-23). Briefly, PCYC-1122e (NCT01500733) was a phase 2, open-label, single-center study evaluating single-agent oral ibrutinib (420 mg once daily until PD or unacceptable toxicity), including in patients with previously untreated CLL with *TP53* aberrations (21). RESONATE-2 (PCYC-1115/16; NCT01722487) was a multicenter, randomized, open-label, phase 3 study comparing ibrutinib (420 mg once daily until PD or unacceptable toxicity) vs chlorambucil in patients aged ≥ 65 years with previously untreated CLL/SLL without del(17p) (22). iLLUMINATE (PCYC-1130; NCT02264574) was a multicenter, randomized, open-label, phase 3 study comparing ibrutinib (420 mg once daily until PD or unacceptable toxicity) plus obinutuzumab (100 mg on day 1, 900 mg on day 2, and 1000 mg on days 8 and 15 in cycle 1, then 1000 mg on day 1 of each 28-day cycle for 6 cycles) vs chlorambucil plus obinutuzumab in patients with previously untreated CLL/SLL aged ≥ 65 years or < 65 years with coexisting conditions (3). RESONATE-17 (PCYC-1117; NCT01744691) was a multicenter, open-label, phase 2 study evaluating single-agent ibrutinib (420 mg once daily until PD or unacceptable

toxicity) in patients with relapsed/refractory CLL/SLL with del(17p).(23) RESONATE (PCYC-1112; NCT01578707) was a multicenter, randomized, open-label, phase 3 study comparing single-agent ibrutinib (420 mg once daily until PD or unacceptable toxicity) vs ofatumumab in patients with relapsed/refractory CLL/SLL (24). Each study was approved by institutional review boards at each participating institution and conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent.

Mutation testing

Mutation testing was performed using peripheral blood samples obtained at standardized timepoints which varied by study, starting with either the last available sample timepoint (for patients without PD) or at the last available sample timepoint before PD (for patients with subsequent PD). In addition, for patients who developed PD, further testing was performed using first available samples obtained at or after PD. Specific timepoints for sample collection in each study are listed in **Supplemental Methods**. The median follow-up time of sample collection is included in the **Results**. After enrichment for CD19+ cells, validated testing for *BTK* and *PLCG2* mutations was performed by commercially available next-generation sequencing with Ion Torrent (Thermo Fisher Scientific, RRID:SCR_008452) at the Ohio State University's James Molecular Laboratory (Columbus, OH). Sensitivity of mutation detection was 0.5% variant allele fraction (VAF) for *BTK* (C481X, T474X) and *PLCG2* (R665W, S707X/A708X, L845X, D993X, and amino acids 1140-1144) mutation hotspots. Sex-normalized VAF for *BTK* (normalized to number of X chromosomes) was calculated as

VAF*1 in males and VAF*2 in females. As previously described,(25) VAF was defined as low subclonal ($\geq 0.5\%$ to $< 25\%$), high subclonal ($\geq 25\%$ to $< 85\%$), or clonal ($\geq 85\%$). To identify the time of first detection of *BTK* C481S mutations, which was the most prevalent variant observed in 93% of samples with any *BTK* mutation (**Supplemental Table 1**), samples obtained at earlier timepoints (3- to 6-month intervals), or as available at varying timepoints, were assessed at the Ohio State University's James Molecular Laboratory by C481S-specific quantitative ddPCR assay (QX200 Droplet Digital PCR System, Bio-Rad Laboratories, RRID:SCR_008426) with a sensitivity of 0.1% VAF (with ≥ 5 mutant events). For previously untreated patients, analyses of VAF and time to first detection of mutations were limited by small numbers of patients with detectable mutations.

TP53 aberrations (17p deletion or *TP53* mutations) were assessed at baseline using standard fluorescence in situ hybridization (FISH) methods for detection of 17p deletion and next-generation sequencing for *TP53* mutations. The threshold for reporting *TP53* mutations was a VAF of $\geq 10\%$ per European Research Initiative on CLL (ERIC) recommendations.

Statistical analysis

Analyses were prespecified in a statistical analysis plan prior to initiation of the project. Mutational frequencies were summarized using descriptive statistics. Time from initiation of ibrutinib to first detection of *BTK* C481S mutations was estimated using the Kaplan-Meier method. Differences in time to first detection of *BTK* C481S mutations

were compared between prespecified subset populations: previously untreated vs relapsed/refractory in all patients, previously untreated vs relapsed/refractory in patients with *TP53* aberrations, all patients with vs without *TP53* aberrations, relapsed/refractory patients with vs without *TP53* aberrations, and 1 vs ≥ 3 prior therapies.

Data availability statement

Requests for access to individual participant data from clinical studies conducted by Pharmacyclics LLC, an AbbVie Company, can be submitted through Yale Open Data Access (YODA) Project site at <http://yoda.yale.edu>.

Results

Patients

Of 388 patients tested, 238 (61%) were previously untreated and 150 (39%) had relapsed/refractory disease (**Table 1**). In patients with relapsed/refractory disease, the median number of prior lines of therapy was 2 (range, 1–12). Forty-nine of 238 (21%) previously untreated patients and 100/150 (67%) relapsed/refractory patients had *TP53* aberrations; 126/238 (53%) previously untreated patients and 54/150 (36%) relapsed/refractory patients had unmutated IGHV (82/238 [34%] and 15/150 [10%] patients, respectively, had mutated IGHV). Of note, a relatively large proportion of patients (previously untreated, 4%; with relapsed/refractory disease, 54%) were missing IGHV testing data. Median follow-up for all patients was 35 months (range, 0–72) without PD at the last available sample and was similar for previously untreated (35

months [range, 0–72]) and relapsed/refractory patients (35 months [range, 1–70]) (**Table 1**).

Mutation rates in patients responding to ibrutinib

For patients who were receiving ibrutinib as first-line therapy, mutations in *BTK* and *PLCG2* were uncommon at this follow-up time, with 3% demonstrating mutations in *BTK*, 2% with mutations in *PLCG2*, and 1% with mutations in both genes at last follow-up without PD (**Figure 1**). Mutations at similar follow-up times as first-line therapy were more common in those with relapsed/refractory CLL, with 30% having evidence of mutations in *BTK*, 7% with mutations in *PLCG2*, and 5% with mutations in both genes at last follow-up without PD (**Figure 1**). Similar rates of mutations were seen in patients with *TP53* aberrations. With median follow-up of 56 months (range, 1–72) for previously untreated patients with *TP53* aberrations, 8% had *BTK* mutations, 2% had *PLCG2* mutations, and 0% had mutations in both genes at last follow-up without PD. With median follow-up of 33 months (range, 1–66) for relapsed/refractory patients with *TP53* aberrations, 28% had *BTK* mutations, 9% had *PLCG2* mutations, and 6% had mutations in both genes at last follow-up without PD. The most commonly detected (by NGS) *BTK* mutations were C481S alone (69/100 samples), C481R-C481S double mutation (7/100), and C481F-C481S-C481Y triple mutation (6/100) (**Supplemental Table 1**). Overall, C481S was observed in 93/100 samples with any *BTK* variant. *BTK* L528 mutation was detected in 3 samples, 2 samples had co-occurring *BTK* C481F, C481R, and C481Y mutations, and 1 sample had a C481R mutation. A list of detected *BTK* mutations is provided in **Supplemental Tables 1 and 2**.

Freedom from BTK mutations in patients responding to ibrutinib

Previously untreated patients remained free from *BTK* C481S mutations significantly longer than patients with relapsed/refractory disease, with a 93% reduction in the risk for development of detectable *BTK* C481S mutations (hazard ratio [HR], 0.060; 95% CI, 0.024–0.153; $P < 0.001$). Median time to first detection of *BTK* C481S mutation was not reached (95% CI, not estimable [NE]) in previously untreated patients, and it was 61 months (95% CI, 47–NE) in patients with relapsed/refractory disease (**Figure 2A**). Three-year mutation-free estimates were 100% vs 80%, respectively. Among patients with relapsed/refractory disease, there was no significant difference in freedom from *BTK* C481S mutation between patients with ≥ 3 vs 1 prior lines of therapy (HR, 0.720; 95% CI, 0.329–1.575; $P = 0.410$) (**Figure 2B**).

In the pooled population, comprising both previously untreated patients and those with relapsed/refractory disease, there was a superior freedom from detection of *BTK* C481S mutations in patients without *TP53* aberrations vs those with *TP53* aberrations (HR, 0.344; 95% CI, 0.194–0.608; $P < 0.001$), with 3-year mutation-free estimates of 97% vs 84% (**Figure 2C**). Among patients with *TP53* aberrations, previously untreated patients had superior freedom from detection of *BTK* C481S mutations compared with patients with relapsed/refractory disease (HR, 0.084; 95% CI, 0.025–0.281; $P < 0.001$), with 3-year mutation-free estimates of 100% vs 73% (**Figure 2D**). Among patients with relapsed/refractory disease, those without *TP53* aberrations had a superior freedom from *BTK* C481S mutations than those with *TP53* aberrations (HR, 0.427; 95% CI,

0.225–0.808; $P=0.009$); 3-year mutation-free estimates were 89% vs 73% (**Figure 2E**).

In contrast, previously untreated patients had identical 3-year mutation-free estimates of 100% for patients with and without *TP53* aberrations.

Patients with clinical PD

At the time of analysis, 17 previously untreated patients and 55 patients with relapsed/refractory disease had known PD, of whom 12 and 45 patients, respectively, were also evaluable for mutations at the time of PD (**Table 2**). *BTK* mutations were detected at PD in 3/12 (25%) previously untreated patients and in 22/45 (49%) of patients with relapsed/refractory disease. *PLCG2* mutations were detected at PD in a single (1/12; 8%) previously untreated patient and in 6/45 (13%) patients with relapsed/refractory disease. Co-occurring *BTK* and *PLCG2* mutations were detected at PD in a single previously untreated patient (1/12, 8%) and in 5/45 (11%) patients with relapsed/refractory disease (**Table 2**). Mutations in the evaluable patients with PD were only detected in individuals on active treatment. No mutations were detected in the 5/12 previously untreated patients and in 4/45 patients with relapsed/refractory disease who had discontinued ibrutinib more than 1 week before PD occurred.

Among evaluable patients who had detectable *BTK* or *PLCG2* mutations at the time of PD, *BTK* mutations were detected prior to clinical PD in 2/3 (67%) previously untreated patients and 19/22 (86%) patients with relapsed/refractory disease. Prior to clinical PD, *PLCG2* mutations were detected in 0/1 (0%) and 3/6 (50%) of such patients, respectively (**Table 2**). Time from first detection of *BTK* mutation to PD was 11.3

months in 1 previously untreated patient who developed PD; the data were not available for the other 2 previously untreated patients with PD. The median time from first detection of *BTK* mutation to clinical PD was 8.5 months (range, 0–35.7 months) among the 23 patients with relapsed/refractory disease with *BTK* mutations who developed PD at any timepoint.

PD with increased lymphocyte counts occurred in a single previously untreated patient (1/238; 0.4%) and in 18/150 (12%) patients with relapsed/refractory disease, while PD with increased lymphadenopathy occurred in 13/238 (5%) and 15/150 (10%) such patients, respectively. In the combined analysis of all patients with PD, *BTK* and *PLCG2* mutations in peripheral blood samples were more frequently associated with the PD criterion of increased lymphocyte counts than with increased lymphadenopathy (**Table 3**). *BTK* mutations were detected at PD in 13/16 (81%) patients with increased lymphocyte counts vs 12/25 (48%) patients with increased lymphadenopathy. *PLCG2* mutations were detected at PD in 3/16 (19%) with increased lymphocyte counts vs 2/25 (8%) patients with increased lymphadenopathy.

Richter's transformation occurred in 2/238 (1%) previously untreated patients and in 18/150 (12%) patients with relapsed/refractory disease. *BTK* and *PLCG2* mutations were also detectable in peripheral blood samples from both types of patients who also had Richter's transformation, but at lower frequencies than in patients who progressed without transformation (**Supplemental Table 3**). At the time of Richter's transformation,

BTK and *PLCG2* mutations were detected in 2/13 (15%) and 1/13 (8%) patients, respectively.

Kinetics of BTK mutation emergence

VAF of *BTK* mutations was highly variable in patients with and without PD (**Figure 3A-D**). In previously untreated patients without PD who had detectable *BTK* C481S mutations (n=4), low VAF mutated subclones were identified in 3 patients (0.6%, 1.1%, 3.6%), while high subclonal VAF at 55% was identified in 1 patient (**Figure 3A**). In patients with relapsed/refractory disease without PD who had detectable *BTK* C481S mutations (n=24), VAF was highly variable, and ranged from 0.5% to 97% (**Figure 3B**). Low VAF mutated subclones ($\geq 0.5\%$ to $< 25\%$) were identified in 14/24 patients, high subclonal VAF ($\geq 25\%$ to $< 85\%$) in 8/24 patients, and clonal VAF ($\geq 85\%$) in 2/24 patients. One previously untreated patient with PD who had a detectable *BTK* C481S mutation was found to have high subclonal VAF (29%) (**Figure 3C**). In patients with relapsed/refractory disease with PD who had detectable *BTK* C481S mutations (n=23), VAF ranged from 0.5% to 97% (**Figure 3D**). Low VAF mutated subclones were identified in 7/23 patients, high subclonal VAF in 10/23 patients, and clonal VAF in 6/23 patients. VAFs fluctuated over time, and the rates of change in VAF also varied between patients with detectable mutations.

Discussion

To our knowledge, this is the largest analysis to date of *BTK/PLCG2* mutations in patients with CLL receiving ibrutinib treatment (N=388). It also provides long-term

follow-up data from patients with diverse clinical risk factors and up to 6 years of continuous ibrutinib treatment (median of 35 months). Consistent with the low frequency of PD and *BTK* and *PLCG2* mutations in previously untreated patients receiving first-line ibrutinib therapy, (10) those mutations were also relatively rare in patients without PD in this study (3% and 2%, respectively). Their higher frequency in patients with relapsed/refractory disease (30% and 7%, respectively) is also consistent with the greater risk of PD for these patients relative to previously untreated patients, with PFS rates of 40% at 5 years (relapsed/refractory) and 61% at 6.5 years (previously untreated) in patients treated with single-agent ibrutinib in the RESONATE and RESONATE-2 studies, respectively (2,26).

To understand the time to development of mutations, we focused on *BTK* C481S variant specifically, which was observed in most samples harboring any *BTK* mutations. Time to development of *BTK* mutations during ibrutinib therapy was longer in previously untreated patients relative to patients with relapsed/refractory disease. Even in the latter group, *BTK* mutations were noted after prolonged ibrutinib treatment, with a median time of 5 years, longer than the median time to initial detection of mutation reported in patients treated with ibrutinib in a phase 2 study (36 months; range, 6–72) (12). The presence of *TP53* aberrations increased the risk of mutation development overall and in patients with relapsed/refractory disease. Interestingly, previously untreated patients with *TP53* aberrations had a significantly reduced risk of mutation development compared to their counterparts with relapsed/refractory disease. This is consistent with reported long-term PFS of up to 61% at 6 years for patients with *TP53* aberrations

receiving first-line ibrutinib (27-29). By contrast, no significant difference was noted when comparing the number of prior lines of therapy in patients with relapsed/refractory disease.

These differences were also observed in patients with clinical PD while on ibrutinib treatment, in whom *BTK* or *PLCG2* mutations were less frequently detected in the previously untreated subset than in those with relapsed/refractory disease. (The caveat is a small number of such patients who were previously untreated.) At the time of PD, *BTK* and/or *PLCG2* mutations were detected in 25% of previously untreated patients, and in 51% of those with relapsed/refractory disease. A similar *BTK* mutation frequency (53%) rates was reported in a recent real-world study of patients with PD while on ibrutinib (30). These mutation rates are notably lower than the values previously reported for patients with CLL progression while on ibrutinib (70%) or acalabrutinib (80%), (9,14,15) which may be explained, at least in part, by differences in patient populations. In particular, our study had lower proportions of patients with advanced Rai stage compared with previously published reports of mutations in patients with PD (9,14,15). Substantial fractions of patients with missing IGHV testing data are also a limitation in understanding the population with relapsed-refractory disease.

Notably, mutation rates were consistent across previous reports, despite variability in the proportion of patients with *TP53* aberrations (15% to 63%), compared with 38% in our study (9,14,15). Although the same mutations in the BTK pathway have been observed with ibrutinib and other covalent BTK inhibitors, acalabrutinib and zanubrutinib, (15,31,32) differences in binding properties may influence the clonal

fitness and therefore the frequency and time to development of various resistance-associated mutations (20,33,34). Consequently, the frequencies of *BTK* and *PLCG2* mutations with ibrutinib may not be directly comparable to those observed with other BTK inhibitors (32).

In the 49% of patients with relapsed/refractory disease in our study who had PD without detectable *BTK* or *PLCG2* mutations, other mechanisms of progression remain to be identified. Such mechanisms may include clonal evolution with acquisition of other driver mutations in CLL. Identification of patients with PD without detectable *BTK* or *PLCG2* mutations may present an opportunity to better understand the biology of CLL progression and improve subsequent treatment options. Although our sample size was relatively small, it should be pointed out that we detected *BTK* mutations up to 36 months before clinical PD. This is longer than the period of 12-15 months, reported previously (9,14).

Consistent with previous reports, (9,14,35) *BTK* or *PLCG2* mutations were less common in patients with Richter's transformation than in those with other forms of CLL progression. This is in line with the finding that *BTK* mutations were generally detected after prolonged treatment with ibrutinib, with a median time to mutation of over 5 years, whereas Richter's transformation typically occurs within the first 2 years of treatment and likely reflects the impact of pre-existing genetic alterations (9,14,36). Since these BCR pathway mutations appear with increasing frequency after prolonged exposure to continuous ibrutinib therapy, novel fixed-duration combination regimens with targeted

agents, such as ibrutinib plus venetoclax, may delay or eliminate the development of resistance. Long-term follow-up of ongoing studies with these regimens will be required to answer this question.

Previous studies suggest a trend of increasing VAF of *BTK* and *PLCG2* mutations over time on ibrutinib therapy, suggesting a growth advantage for clones harboring such mutations (9,14,35). In our study, VAF generally showed similar increases but kinetics of emergence were highly variable and fluctuated over time in individual patients. These observed variations may be due in part to limitations inherent to intent-to-treat analyses, such as missing or incomplete data. The small number of patients with PD with evaluable mutations and varying rates of mutation testing at baseline further contribute to these fluctuations. Moreover, similar to previous studies, (9,14,35) it is possible that peripheral blood sampling may not fully represent the clonal evolution taking place within lymph nodes or other compartments where clonal evolution may contribute to PD in patients with low peripheral blood VAF and in those with increased lymphadenopathy. Prior studies have also established that these mutations are not universally present in patients with CLL who are developing disease progression while receiving ibrutinib. The subclonal status of these mutations, which are generally present at PD, does not fully explain resistance, and the potential mechanisms that allow cooperation between mutant and wild-type clones are yet to be elucidated. Data on VAF, on the delay from mutation detection to the occurrence of PD, on the frequency of detectable *BTK* or *PLCG2* mutations in patients without PD, and on the variability in presence of these mutations at PD, indicate that the presence of *BTK* or *PLCG2* mutations may not

reliably predict the time to onset of PD in individual patients. However, these data do provide a basis for considering the value of such testing. Patients with known *BTK* or *PLCG2* mutations might benefit from closer monitoring for PD, and future investigations may yield regimens geared toward such patients, for example biomarker-driven treatment intensification.

In conclusion, these long-term data from ibrutinib-treated patients with or without PD, with a median follow-up of 35 months, have demonstrated the relative rarity of *BTK* and *PLCG2* mutations in the first-line setting, including in patients with *TP53* aberrations. The importance of *TP53* aberrations in the development of these mutations in patients with relapsed/refractory disease is also established. Finally, the presence of these mutations in up to one-third of ibrutinib-treated patients with relapsed/refractory CLL and without PD suggest that individuals harboring these mutations continue to benefit from ibrutinib until progression occurs. These data support CLL/SLL consensus guidelines, which recommend that the detection of *BTK* or *PLCG2* mutations alone does not currently warrant changes in treatment in the absence of PD, and also highlights an opportunity to develop clinical trials designed to prolong clinical remissions for patients receiving long-term BTK inhibitor treatment.

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Authorship Contributions

J.A.W., J.P.D., D.F.J., and A.W. designed the analysis; J.A.W., P.G., J.C.B., I.E.A., C.M., S.O., D.J., and A.W. contributed to data collection; L.W.K.C., E.C., and K.K. performed the data analyses; E.C. and J.P.D. confirmed the accuracy of the data and compiled it for analysis; J.A.W. and J.P.D. collaboratively wrote the first draft of the manuscript; all authors had access to the data and were involved in the interpretation of data, contributed to the manuscript review and revisions, and approved the final version for submission.

Disclosure of Conflicts of Interest

J.A.W. reports a consulting/advisory role with AbbVie, AstraZeneca, ArQule, BeiGene, Genentech, Janssen, Loxo, Newave, and Pharmacyclics LLC, an AbbVie Company; and has received research funding from AbbVie, Loxo, Karyopharm, MorphoSys, Schrodinger, and Verastem. **P.G.** reports honoraria from and a consulting/advisory role with AbbVie, AstraZeneca, ArQule/Merck Sharp & Dohme, Celgene/Juno/Bristol Myers Squibb, Janssen, Loxo/Lilly, MEI Pharma and Roche; and has received research

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employment with Pharmacyclics LLC, an AbbVie Company, and Biosplice, and current employment with Kartos Therapeutics; and has stock ownership in Biosplice, Gilead, Kartos Therapeutics, and AbbVie. **D.F.J.** reports former employment and leadership roles with Pharmacyclics LLC, an AbbVie Company and current employment and leadership roles with Summit Therapeutics LLC; has stock ownership in AbbVie and Summit Therapeutics LLC; and has patents, royalties, or other intellectual property with Pharmacyclics LLC, an AbbVie Company. **A.W.** has received research funding from Acerta, Genmab, Merck, Nurix, Verastem, and Pharmacyclics LLC, an AbbVie Company.

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Tables and Figures

Table 1. Baseline characteristics and follow-up on study

Characteristic	Previously untreated <i>n</i> = 238	Relapsed/ refractory <i>n</i> = 150	All patients <i>N</i> = 388
Median follow-up (range), months	35 (0–72)	36 (1–70)	35 (0–72)
Median age (range), years	71 (39–89)	65 (44–86)	69 (39–89)
Bulky disease (≥5 cm), <i>n</i> (%)			
Yes	76 (32)	88 (59)	164 (42)
No	161 (68)	61 (41)	222 (57)
Missing	1 (<1)	1 (1)	2 (1)
IGHV, <i>n</i> (%)			
Unmutated	126 (53)	54 (36)	180 (46)
Mutated	82 (34)	15 (10)	97 (25)
No amplification	18 (8)	0	18 (5)
Polyclonal	3 (1)	0	3 (1)
Missing	9 (4)	81 (54)	90 (23)
del(11q), <i>n</i> (%)			
Yes	41 (17)	40 (27)	81 (21)
No	192 (81)	106 (71)	298 (77)
Missing	5 (2)	4 (3)	9 (2)
del(17p)/ <i>TP53</i> mutated, <i>n</i> (%)			

Yes	49 (21)	100 (67)	149 (38)
No	180 (76)	42 (28)	222 (57)
Missing ^a	9 (4)	8 (5)	17 (4)
Median prior lines of therapy, (range)	NA	2 (1–3)	—
Number of prior lines of therapy			
0	238 (100)	NA	238 (61)
1	NA	44 (29)	44 (11)
2	NA	50 (33)	50 (13)
≥3	NA	56 (37)	56 (14)
Prior chemotherapy or chemoimmunotherapy, n (%)	NA	133 (89)	—

NA, not applicable.

^aPatients had missing data for 1 or both tests for del(17p) by FISH or *TP53* mutational status.

Table 2. *BTK* and *PLCG2* mutations in patients with PD^a, n/N or n/N (%)

	Previously untreated	Relapsed/ Refractory CLL
Patients with known PD as of testing data cuts	17/238 (7)	55/150 (37)
Patients evaluable for mutations at PD	12/238 (5)	45/150 (30)
Patients with <i>BTK</i> mutation present at PD	3/12 (25)	22 ^b /45 (49)
<i>BTK</i> mutation detected prior to PD	2/3	19/22
<i>BTK</i> mutation not detected prior to PD	1/3	3/22
Patients with <i>PLCG2</i> mutation present at PD	1/12 (8) ^c	6/45 (13) ^d
<i>PLCG2</i> mutation detected prior to PD	0	3/6
<i>PLCG2</i> mutation not detected prior to PD	1/1	3/6

PD, progressive disease.

^aTesting was performed using the first available samples obtained at or after PD.

^bOne additional patient had *BTK* mutation detected prior to clinical PD, but there were no additional samples available to confirm the *BTK* mutation status at or after PD.

^cOne patient with *PLCG2* mutation also had co-occurring *BTK* mutation.

^dFive patients with *PLCG2* mutation also had co-occurring *BTK* mutation.

Table 3. Frequency of *BTK* and *PLCG2* mutations in previously untreated patients or those with relapsed/refractory CLL and PD, based on increased lymphadenopathy or increased blood lymphocytes per iwCLL criteria^a, n/N or n/N (%)

	Increased lymph-adenopathy <i>n</i> = 28	Increased blood lymphocytes <i>n</i> = 19
Patients with known PD as of testing data cuts	28 (100)	19 (100)
Patients evaluable for mutations at PD	25 (89)	16 (84)
Patients with <i>BTK</i> mutation present at PD	12/25 (48)	13/16 (81)
<i>BTK</i> mutation detected prior to PD	9/12	11/13
<i>BTK</i> mutation not detected prior to PD	3/12	2/13
Patients with <i>PLCG2</i> mutation present at PD	2/25 (8)	3/16 (19)
<i>PLCG2</i> mutation detected prior to PD	0	2/3
<i>PLCG2</i> mutation not detected prior to PD	2/2	1/3

CLL, chronic lymphocytic leukemia; PD, progressive disease.

^aTesting was performed using the first available samples obtained at or after PD.

Figure Legends

Figure 1. *BTK* and *PLCG2* mutation rates in patients responding to ibrutinib.

Testing was performed in the last available sample for patients without PD, or the last sample before PD for patients with subsequent PD.

1L, first-line; PD, progressive disease; R/R, relapsed/refractory.

Figure 2. Kaplan-Meier estimates of freedom from *BTK* C481S mutations. (A)

Previously untreated patients vs patients with relapsed/refractory disease, (B) patients with relapsed/refractory disease with 1 vs ≥ 3 prior lines of therapy, (C) patients with vs without *TP53* aberrations (D) previously untreated patients vs patients with relapsed/refractory disease with *TP53* aberrations, and (E) patients with relapsed/refractory disease with vs without *TP53* aberrations.

1L, first-line; HR, hazard ratio; NE, not estimable; NR, not reached; R/R, relapsed/refractory.

Figure 3. Kinetics of *BTK* C481S mutation emergence. Sex-normalized VAF over time in (A) previously untreated and (B) relapsed/refractory patients without PD (from start of ibrutinib treatment); (C) previously untreated and (D) relapsed/refractory patients with PD (relative to the date of PD). Patients evaluated is the number of patients with valid samples within 100 days of the landmark time point. Sex-normalized VAF (normalized to number of X chromosomes) were calculated as $VAF \times 1$ in males and $VAF \times 2$ in females. Red lines indicate male patients; blue lines indicate female patients; * indicates VAF >100% as a result of sex normalization.

1L, first-line; PD, progressive disease; R/R, relapsed/refractory; VAF, variant allele fraction.

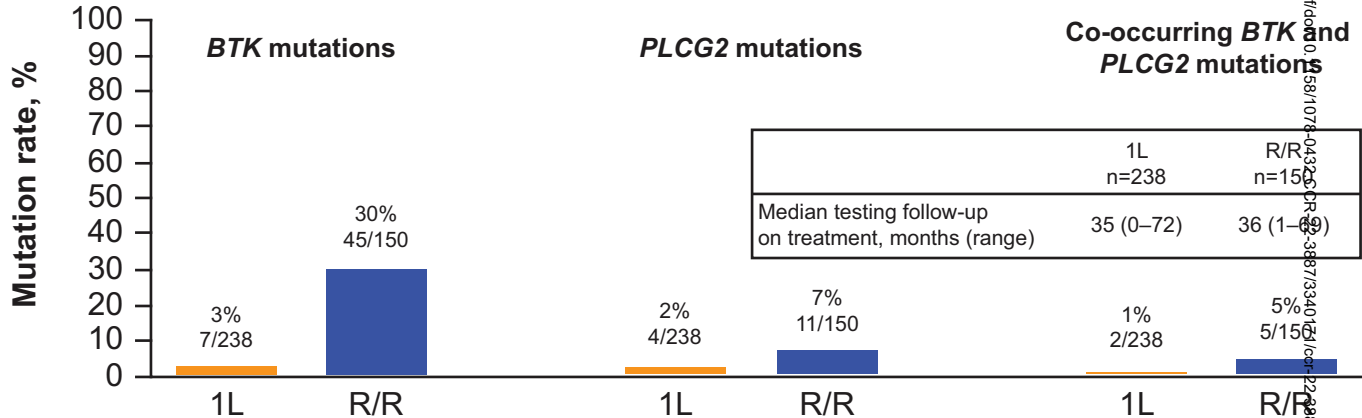
^a5/221 patients had VAF >0% at any time point.

^b25/95 patients had VAF >0% at any time point.

^c1/17 patients had VAF >0% at any time point.

^d23/55 patients had VAF >0% at any time point.

Figure 1



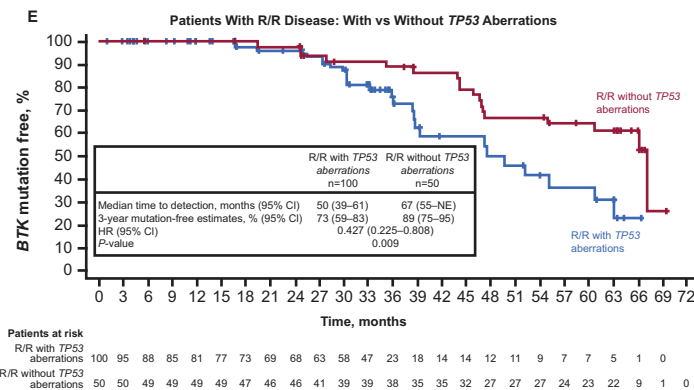
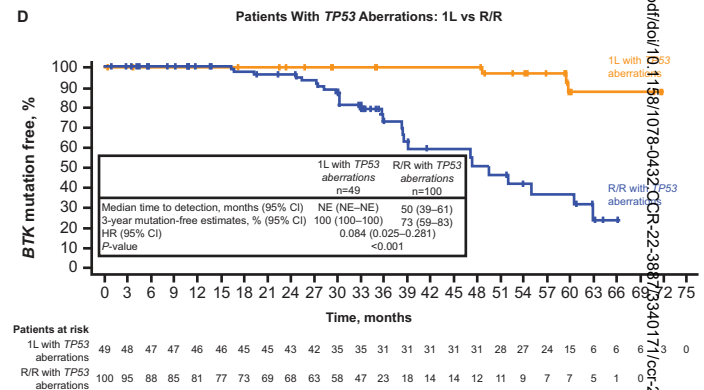
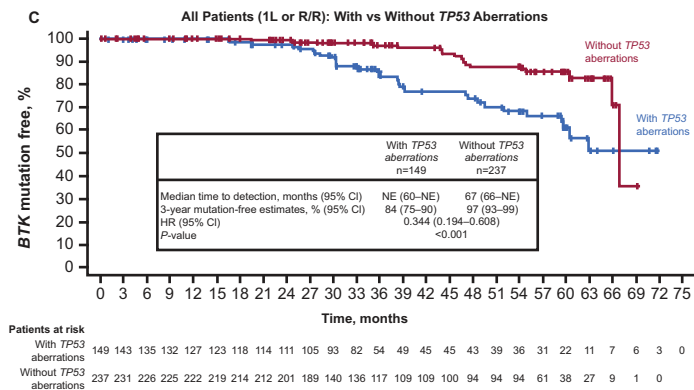
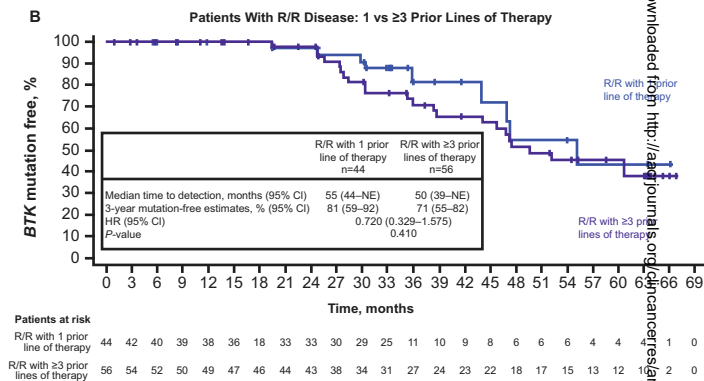
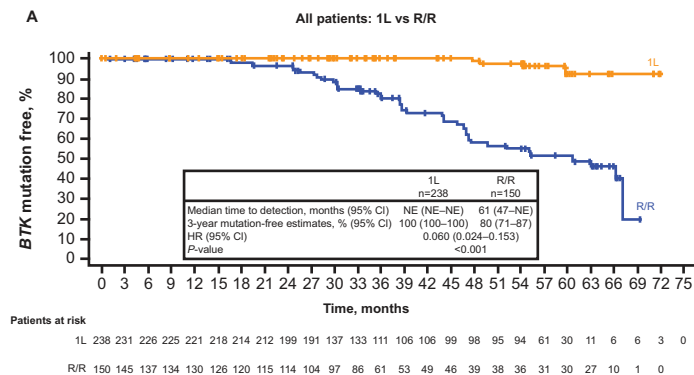


Figure 3