LYMPHOID NEOPLASIA

Hypermorphic mutation of phospholipase C, $\gamma 2$ acquired in ibrutinib-resistant CLL confers BTK independency upon B-cell receptor activation

Ta-Ming Liu,¹ Jennifer A. Woyach,¹ Yiming Zhong,¹ Arletta Lozanski,¹ Gerard Lozanski,² Shuai Dong,¹ Ethan Strattan,¹ Amy Lehman,³ Xiaoli Zhang,³ Jeffrey A. Jones,¹ Joseph Flynn,¹ Leslie A. Andritsos,¹ Kami Maddocks,¹ Samantha M. Jaglowski,¹ Kristie A. Blum,¹ John C. Byrd,¹ Jason A. Dubovsky,¹ and Amy J. Johnson¹

¹Department of Internal Medicine, Division of Hematology, and ²Department of Pathology, Wexner Medical Center, The Ohio State University, Columbus, OH; and ³Center for Biostatistics, The Ohio State University, Columbus, OH

Key Points

- Hypermorphic PLCγ2 is independent of BTK activation.
- SYK or LYN inhibition antagonizes mutant PLCγ2mediated signaling events.

Ibrutinib has significantly improved the outcome of patients with relapsed chronic lymphocytic leukemia (CLL). Recent reports attribute ibrutinib resistance to acquired mutations in Bruton agammaglobulinemia tyrosine kinase (*BTK*), the target of ibrutinib, as well as the immediate downstream effector phospholipase C, γ^2 (*PLCG2*). Although the C481S mutation found in *BTK* has been shown to disable ibrutinib's capacity to irreversibly bind this primary target, the detailed mechanisms of mutations in *PLCG2* have yet to be established. Herein, we characterize the enhanced signaling competence, BTK independence, and surface immunoglobulin dependence of the *PLCG2* mutation at R665W, which has been documented in ibrutinib-resistant CLL. Our data demonstrate that this missense alteration

elicits BTK-independent activation after B-cell receptor engagement, implying the formation of a novel BTK-bypass pathway. Consistent with previous results, *PLCG2*^{R665W} confers hypermorphic induction of downstream signaling events. Our studies reveal that proximal kinases SYK and LYN are critical for the activation of mutant *PLCG2* and that therapeutics targeting SYK and LYN can combat molecular resistance in cell line models and primary CLL cells from ibrutinib-resistant patients. Altogether, our results engender a molecular understanding of the identified aberration at *PLCG2* and explore its functional dependency on BTK, SYK, and LYN, suggesting alternative strategies to combat acquired ibrutinib resistance. (*Blood.* 2015;126(1):61-68)

Introduction

Chronic lymphocytic leukemia (CLL) is a clonally derived mature B-cell malignancy characterized by microenvironment dependence and constitutive activation of the B-cell receptor (BCR) pathway.¹⁴ Conventional therapy for fit, young patients includes chemoimmunotherapy with fludarabine, cyclophosphamide, and rituximab.^{5,6} Outside of allogeneic stem cell transplantation, CLL is considered an incurable disease, and patients will eventually relapse following front-line therapy.

Recent advances in CLL disease biology confirm that the malignant cells receive heterogenic multicellular stimuli within the lymphoid tissues that subvert apoptosis and promote proliferation.^{3,7} Despite profound interpatient heterogeneity, the BCR pathway is a critical axis of CLL survival signaling, given its dominant role in nearly all cases of CLL.^{4,8} Mutations of the immunoglobulin heavy-chain variable region (IGVH) gene in CLL are associated with favorable responses, whereas unmutated subsets of CLL predict poor prognosis.^{9,10} Current studies demonstrate anergic features in mutated CLL leading to down-modulation of surface immunoglobulin M (IgM), whereas unmutated CLL could transmit positive signaling and promote disease progression.¹¹ Hence, novel targeted therapies aimed to abrogate BCR-integral kinases have demonstrated marked clinical activity.¹² Ibrutinib

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is one such BCR-targeted therapy that covalently binds Bruton agammaglobulinemia tyrosine kinase (BTK) at cysteine 481. This prevents the critical LYN and SYK directed autophosphorylation of its Src homology 3 (SH3) domain; thereby preventing downstream activation of PLCy2 and, in turn, blocking downstream BCR signaling. Nevertheless, ibrutinib resistance due to acquired mutations in BTK and its direct downstream effector PLC γ 2 has been documented in a small number of cases.¹³ Whereas the C481S mutation in BTK prevents ibrutinib covalent binding and thereby converts this agent to a less effective reversible inhibitor,¹³ the previously identified R665W and L845F in PLC γ 2 have been only partially characterized and it remains unclear how these mutations confer molecular resistance. Although it is clear that these mutations are hypermorphic and augment BCR downstream signaling events regardless of BTK activity, we have shown previously that they do not induce autonomous PLCy2 signaling and still rely on BCR signaling events. Given that conventional activation of PLCy2 requires the cooperation of BTK,¹⁴ the underlying molecular mechanisms that allow these PLCy2 mutations to bypass BTK inhibition remain an important question when considering new therapeutic strategies to combat ibrutinib resistance.

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T.-M.L., J.A.W., Y.Z., J.A.D., and A.J.J. contributed equally to this study.

Table 1. Relapsed patient information with R665W mutation in <i>PL</i>	.CG2
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Number	Patient ID	Days on ibrutinib	AA change	Baseline		Relapse			
				Coverage	Variant frequency (%)	Coverage	Variant frequency (%)	lgVH	Del(17p)
<i>R</i> -1	0002	511	R665W	870	0	9977	5.5	U	NA
R-2	1140*	505	R665W	3465	0	2781	2	U	Positive
R-3	0605	673	R665W	3230	0	9252	45	М	Positive

AA, amino acid; M, mutated status of IgVH; NA, non-detected, U, unmutated status of IgVH.

*Also had acquired C481S mutation in BTK at relapse.

The BCR is composed of a tetrameric complex of Ig heavy and light chains, including Ig α (CD79 α) and Ig β (CD79 β), in which immunoreceptor tyrosine-based activation motifs are harbored. Upon BCR engagement, Src-family protein tyrosine kinases, primarily LYN, as well as SYK and BTK are activated by tyrosine phosphorylation upon receptor aggregation,¹⁵ thereby transducing downstream signals. PLC γ 2 is responsible for hydrolyzing phosphatidylinositol to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) upon activation. Whereas IP₃ mediates Ca²⁺ mobilization, DAG activates protein kinase C family members.¹⁶ Previous evidence indicates defective responses to α-IgM in Plcg2 knockout mice,¹⁷ a similar phenotype observed in BTK-deficient B cells,¹⁴ suggesting that BTK and PLC γ 2 are prerequisite molecules within the BCR activation pathway. In addition, defective IP₃ production and calcium mobilization have been described in SYK-deficient DT40 cells,¹⁸ showing that SYK activation is indispensable for coupling BCR signaling to PLCy2 activation. The adaptor function of B-cell linker protein (BLNK) for recruiting both BTK and PLCy2 to the membrane compartment has been illustrated,¹⁹ indicating a scenario in which SYK activates BTK leading to downstream PLCy2 activation. Until recently, the functional requirement of LYN in PLC γ 2 activation was still unclear. In regard to BCR activation, LYN-mediated Iga-IgB phosphorylation is essential for SYK activation.²⁰ The direct association between LYN and PLC γ 2 has been reported,²¹ however, LYN-null DT40 cells reveal a differential response in calcium release and IP₃ production,¹⁸ suggesting that LYN may jointly activate PLC γ 2 in coordination with SYK, BTK, and BLNK.

Given the direct clinical need, we sought to investigate the molecular pathogenesis of the acquired PLC γ 2 mutation in ibrutinib resistance. Ion torrent sequencing analysis from additional relapsed patients (in some patients without the coexistence of BTK mutations) confirmed the previously identified R665W mutation at PLC γ 2. The R665W mutation in *PLCG2*, which confers BTK independence and molecular resistance to ibrutinib was evaluated for its hypermorphic capacity to impel enhanced downstream signaling and Ca²⁺ flux in the setting of BCR stimulation. Furthermore, we observed the functional dependence of the R665W mutation SYK and LYN, which cooperate with mutant PLC γ 2 to form a BTK bypass pathway. Together, our findings provide additional insight into the underlying mechanisms of ibrutinib resistance, and identify possible alternative therapies that target this aberration.

Materials and methods

Ion torrent analysis

DNA was extracted from cryopreserved isolated CLL cells using a DNA extraction kit (QIAamp DNA Mini Kit, Qiagen) according to the manufacturer's recommendations. DNA was quantified using a spectrophotometric method (NanoDrop 2000, Thermo Scientific) using the standard 260/280 optical density

ratio. Analysis of the PLCG2 gene was performed using next-generation sequencing Ion Torrent platform and reagents from Life Technologies (Carlsbad, CA). Library was prepared with Ion AmpliSeq Library Kit 2.0 with a custom designed panel of AmpliSeq primers (panel design IAD48992, pipeline version 3.0, 87 amplicons in 2 pools, 17 kb panel size, 99.68% coverage and/or IAD35546 panel, pipeline version 1.2 amplicons in 2 pools, 48 kb panel size, 98.9% coverage). Both panels cover the entire coding sequence and intronic splice acceptor and donor sites for PLCG2. For multiple samples, we used IonExpress barcode adapters. DNA was amplified on the GeneAmp PCR system 9700 Dual 96-well Thermal Cycler from Applied Biosystems. Polymerase chain reaction (PCR) product was purified with the Agencourt AMPure XP Kit (Beckman Coulter, Indianapolis, IN). Library was quantified using real-time PCR with an Ion Library TAQMAN Quantitation Kit on Applied Biosystems ViiA 7 Real-Time PCR System to allow optimal final dilution of library for template preparation on OneTouch OT2 version instrument with Ion PGM Template OT2 200. The ion sphere particles enrichment and purification was performed on the Ion OneTouch 2 enrichment system. Purified iron sphere particles were analyzed on the Ion Torrent Personal Genome Machine using IonPGM Sequencing 200 version 2 Kit and 316/318 chips version 2. Data were collected and analyzed using the Torrent Server with Torrent Suite 4.0.2 version. Final analysis of sequence data was performed using a combination of software: VariantCaller version 4.0-r76860, Ion Torrent IGV3.6.033, and Ion Reporter version 4.0. The NM002661.3 reference sequence was used for analysis. The entire length of sequences was reviewed manually using these programs to assess for deviation from reference sequence, and to evaluate the quality of sequence and the depth of coverage. The depth of coverage ranged from 1000 to 15 000 for different amplicons.

Reagents and antibodies

Antibodies against phospho-AKT (Ser473), AKT, phospho-extracellular signalregulated kinase (p-ERK)1/2 (Thr202/Tyr204), ERK1/2, phospho-PLC γ 2 (Tyr1217), PLC γ 2, phospho-SYK (Tyr525/526), BTK, phospho-Src/LYN, and LYN were obtained from Cell Signaling Technologies (Danvers, MA). Phospho-BTK (Tyr223) was obtained from Abcam (Cambridge, MA), SYK (N-19) from Santa Cruz Biotechnology (Santa Cruz, CA), and glyceraldehyde-3-phosphate dehydrogenase from Millipore (Billerica, MA).

PLCG2 cloning and cell culture

The chicken DT40 cell lines were provided by the RIKEN BioResource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan. Cells were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) with 2 mM L-glutamine, 10% fetal bovine serum, and 56 U/mL penicillin-streptomycin (Life Technologies). Human PLCG2 (BC007565.1) was cloned into a pBABE-Puro vector. Mutation in *PLCG2* at R665W was derived using site-directed mutagenesis (QuikChange Lightning, Stratagene-Agilent Technologies, Santa Clara, CA). The pBABE-PLCG2 vectors were retrovirally introduced into cells. Cells stably expressing variant PLC γ 2 were selected out in the presence of 1 µg/mL puromycin for 2 weeks.

Immunoblot assays

Total lysate was extracted in M-PER Mammalian Protein Extraction Reagent (Pierce). Equivalent amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blots were probed with the appropriate primary and horseradish peroxidase-conjugated secondary antibodies and developed with chemiluminescent Figure 1. A diagram of the human *PLCG2* gene. Depicts encompassed domains of human *PLCG2*. R665W and S707Y mutations identified in relapse CLL are harbored within the SH2 domain. PH, Pleckstrin homology. C-2, calcium binding motif; SH2, Src homology 2; SH3, Src homology 3; X-box, phosphatidylinositol-specific phospholipase C X domain; Y-box, phosphatidylinositol-specific phospholipase C Y domain.



Calcium flux assays

Intracellular calcium change was measured by Calcium Assay Kit (BD Biosciences, San Diego, CA). Briefly, 5×10^4 cells in 50 µL complete RPMI medium were harvested in 96 wells half-area microplates. An equal volume of 1X Dye-loading Solution was added to each well and incubated for 1 hour at 37°C. After incubation at room temperature for another hour, calcium release was measured with the Beckman Coulter DTX880 Microplate Reader. After the acquisition to determine the baseline, 3 µg/mL anti-chicken IgM (SouthernBiotech, Birmingham, AL) was added to stimulate the cells. The areaunder-curve (AUC) was calculated and normalized to samples treated with dimethylsulfoxide (DMSO) alone; 1 µM ibrutinib for 1 hour followed by washout, 2.5 µM R406, 0.5 µM GS-9973, and 0.1 µM dasatinib were used.

Statistical methods

IP-One and calcium flux (AUC) comparisons between relevant conditions (eg, R665W vs wild-type [WT], ibrutinib vs DMSO) were performed using linear



mixed effects models to account for correlations among replicates from the same subject/experiment; data from calcium flux experiments were log-transformed. For experiments with multiple comparisons, *P* values were adjusted using the Holm procedure to control overall type I error at .05. All analyses were performed using SAS/STAT software version 9.3 (SAS Institute Inc., Cary, NC).

Results

Missense point-mutation at R665W in *PLCG2* was identified in relapsed patients

We have previously reported that relapse during ibrutinib therapy is associated with the acquisition of specific gene mutations.^{13,22} Besides C481S at BTK that directly impairs the targeting effect of ibrutinib, our previous studies identified somatically acquired *PLCG2* variants and illustrated how those mutations augment downstream signaling in response to BCR engagement. Remarkably, R665W was recognized in



Figure 2. Hypermorphic mutation R665W acquires resistance to ibrutinib and functions independently to BTK. (A) Downstream phospho-protein activation upon 0.5 µ.g/ml α-IgM stimulation for 15 minutes was examined by western blots in comparison with WT PLCv2 to B665W mutation in DT40 cells (B) 293T cells were retrovirally transduced with WT PLC γ 2 or R665W mutation. The production of IP3 upon 150 ng/ml epidermal growth factor stimulation was measured by IP1 (surrogate of IP₃) accumulation using IP-One ELISA Kit (n = 3 repeated experiments). (C) Calcium flux in BTK-deficient DT40 lines introduced with WT PLC₂2 or R665W mutant were examined. Data represents the AUC from 6 replicates upon 3 μ g/ml α -IgM stimulation, and (D) downstream phospho-protein activation was examined by western blots. DMSO, dimethylsulfoxide; KO, knockout; Un., untreated.



Figure 3. SYK inhibition abrogates PLCG2^{R665W} induced downstream activation. (A) Calcium influx induced by 3 µg/ml α-lgM stimulation was measured in PLCγ2^{-/-} DT40 stably expressed with either WT human PLCG2 or R665W mutant. The data represents AUC by 6 replicates. In the treatment settings, 1.0 µM ibrutinib was pretreated for 1 hour followed by washout; and 0.5 µM GS-9973 or 2.5 µM R406 was treated continuously. **P* < .05; ****P* < .001; NS = *P* > .05. (B) Downstream signaling was examined in PLCγ2^{-/-} DT40 expressing either WT PLCG2 or R665W mutant. Cells were treated with 0.5 µg/ml α-lgM for 15 minutes; 1.0 µM ibrutinib was pretreated for 1 hour followed by washout; and 0.5 µM GS-9973 or 2.5 µM R406 was treated.

3 individual relapsed patients (Table 1). In all patients, the aberration was acquired during therapy, as deep sequencing of baseline samples did not reveal this mutation. Moreover, in 2 resistant patients, *PLCG2* R665W exists without the BTK C481S mutation, indicating that this mutation can lead to resistance without cooperating BTK or other PLCG2 mutations. The detailed characteristics of patients who acquired the R665W mutation are summarized in Table 1.

Hypermorphic PLC $\gamma 2^{R665W}$ mutation is characterized by the ability to amplify BCR downstream signaling

The S707Y variant in human *PLCG2* has been documented as an inherited mutation resulting in pathologic autoimmunity, suggesting that aberrations within SH2 loci of the auto-inhibitory domain may reinforce the enzymatic activity of PLC $\gamma 2$.²³ Given that R665W and S707Y are harbored at the same region (Figure 1), a similar capability was hypothesized. To evaluate the functional consequence of R665W, a chicken-derived PLC $\gamma 2$ -deficient DT40 cell line was stably introduced with either WT or mutant human PLC $\gamma 2$ and stimulated by α -IgM. PLC $\gamma 2$ -deficient DT40 fails to respond to anti-IgM stimulation, whereas the introduction of human PLC $\gamma 2$ can restore the signaling event (see supplemental Figure 1 on the *Blood* Web site). Consistent with previous results,¹³ we found that PLC $\gamma 2^{R665W}$ augments calcium flux, and this effect is resistant to ibrutinib treatment compared with WT (supplemental Figure 2). Also, the R665W variant elicited robust downstream *p*-ERK activation after α -IgM stimulation (Figure 2A),

whereas the WT was sufficiently blocked by ibrutinib. PLC γ 2 cleaves its membrane-bound substrate to generate IP₃ and DAG. IP₃ causes calcium influx from the endoplasmic reticulum and triggers protein kinase C signaling. To examine whether PLCG2^{R665W} potentiates IP₃ production, 293T cells were stably introduced with either WT or PLCG2^{R665W} (supplemental Figure 3). IP₃ production measured by the accumulation of IP₁ was elevated in PLCG2^{R665W} (Figure 2B), implicating that R665W mutation acquires hyperactivity by enhancing downstream signaling. These data suggest that the R665W, similar to the previously identified S707Y mutation,²⁴ confers hypermorphic activity upon BCR activation despite upstream BTK inhibition. Notably, the hypermorphic PLC γ 2 mutant fails to retain phosphorylation of Tyr 1217 after ibrutinib treatment despite preserving the capacity to elicit downstream signaling (Figure 2A).

Hyperactivated PLC γ 2 no longer requires BTK in the BCR signaling pathway

Given that PLC γ 2 is the immediate downstream effector of BTK upon BCR activation, and our results indicate that ibrutinib does not inhibit downstream BCR signaling in the presence of mutated PLC γ 2, we sought to investigate the functional role of BTK in hypermorphic PLC γ 2. BTK-deficient DT40 cells were introduced with either WT or R665W PLCG2 followed by α -IgM stimulation. Consistent with previous results that BTK^{-/-} DT40 cells were nonresponsive to BCR engagement,¹⁴ overexpression of WT human PLCG2 in BTK^{-/-} DT40

Figure 4. LYN suppression abrogates PLCG2^{Re65W}mediated downstream activation. (A) Calcium flux induced by 3 µg/ml α-lgM stimulation was measured in PLC $\gamma 2^{-I}$ DT40 expressing WT human PLCG2 or Re65W mutant. The data represents AUC by 6 replicates. In the treatment settings, 1.0 µM ibrutinib was pretreated for 1 hour followed by washout; 0.1 µM dasatinib was used. **P* < .05; ****P* < .001; NS = *P* > .05. (B) Downstream signaling was accessed in PLC $\gamma 2^{-I-}$ DT40 expressing either WT human PLC $\gamma 2$ or Re65W mutant. Cells were treated with 0.5 µg/ml α-lgM; 1.0 µM ibrutinib was pretreated for 1 hour followed by washout; and 0.1 µM dasatinib was used.



cells triggered only modest calcium flux, and PLCG2^{R665W} significantly enhanced BTK-independent calcium release (Figure 2C) as well as downstream ERK activation (Figure 2D), suggesting that this hypermorphic PLC γ 2 mutant functionally bypasses BTK and promotes downstream activation of the BCR pathway.

Since BTK is not required for triggering activation of PLCG2^{R665W}, we hypothesized that specific upstream kinases, which are attributed to PLC γ 2 activation may still be required, and that targeting these kinases could represent an alternative strategy for the treatment of acquired ibrutinib resistance. Previous studies confirm that SYK-deficient DT40 cells display abolished IP₃ or Ca²⁺ production after BCR engagement.¹⁸ Furthermore, SYK has been shown to directly participate in PLC₂2 activation via interaction with BLNK,⁸ implicating SYK as a possible therapeutic target. To assess this hypothesis, SYK inhibitors were tested against the PLC γ 2 mutant. GS-9973 is one of the SYK inhibitors undergoing clinical evaluation for patients with relapsed or refractory hematologic malignancies. R406 is the active derivative of the prodrug fostamatinib that has been assessed in patients with lymphoma or rheumatoid arthritis. Optimal doses of these agents were assessed in DT40 cells to sufficiently abrogate BCR activation without inducing cytotoxicity (supplemental Figure 4). Remarkably, single agent treatment of either GS-9973 or R406 sufficiently impeded the

hypermorphic calcium release and downstream ERK activation in PLC γ 2-deficient DT40 cells expressed PLCG2^{R665W}, in contrast to ibrutinib, which is only effective in WT (Figure 3A-B). Similar results using combinational treatments compared with the SYK inhibitor alone was observed, demonstrating that SYK proximately functions to BTK/PLC γ 2. These data suggest a new therapeutic role for SYK-targeting in CLL patients with acquired hypermorphic PLC γ 2^{R665W}.

The BCR also utilizes Src-family protein tyrosine kinases like LYN to regulate downstream effectors. Although the functional correlation between LYN and PLC γ 2 has not been fully established, reduced activation of PLC γ 2 and Ca²⁺ production has been characterized in LYN-deficient B cells leading to the assumption that hypermorphic PLC γ 2 may be dependent upon LYN activation.¹⁸ To test this hypothesis, we used dasatinib, a known Src and LYN inhibitor. Our data reveal that PLCG2^{R665W}-induced calcium release and downstream activation could be attenuated by dasatinib (Figure 4A-B). These findings demonstrate the therapeutic potential of LYN inhibition in patients with ibrutinib-resistant PLCG2 mutations, as well as LYN's involvement in regulating PLC γ 2 activation.

SYK and LYN targeting blocks BCR signaling in ibrutinib-resistant CLL samples

To investigate the effects of targeting SYK and LYN, CLL cells from 3 ibrutinib resistant patients bearing $PLCG2^{R665W}$ were tested. Although modest elevation of Ca²⁺ influx in nonresistant samples was



Figure 5. Hyperactive downstream signaling in relapse CLL acquired PLC₂ variants can be blocked by targeting SYK or LYN. (A) Calcium flux from 3 individual relapse CLL (R) bearing mutated PLCy2 or 12 nonresistant CLL samples (NR) were measured, and 5E6 cells per well in 96-half well microplates were stimulated with 10 $\mu\text{g/ml}$ $\alpha\text{-lgM}$ at 37°C for 15 minutes, and then measured by BD Calcium Assay Kit. The data represent AUC from 7 and 12 replicates in resistant and nonresistant samples, respectively; 1.0 µM ibrutinib was pretreated for 1 hour followed by washout. (B) The data represents calcium release by 3 individual relapse CLL (R) or the mean of 12 nonresistant samples (NR) stimulated with 10 µg/ml α -IqM in the presence of 0.5 μ M GS-9973, 2.5 μ M R406, or 0.1 μM dasatinib; 1.0 μM ibrutinib was pretreated for 1 hour followed by washout. ***P < .001. (C) The downstream phospho-protein activation was accessed in CLL cells from a single patient (patient #2 in Table 1) in the baseline or relapse setting. The detailed information of the 3 relapsed CLL patient samples analyzed here are listed in Table 1.

measured, all 3 ibrutinib-resistant CLL samples induced robust Ca^{2+} release upon stimulation (Figure 5A), and this effect was significantly inhibited by dasatinib, GS-9973, or R406 treatment (Figure 5B). Moreover, SYK or LYN inhibition reduced Ca^{2+} flux more effectively than ibrutinib in these resistant samples, reducing overall activation to levels within the range of nonresistant samples (Figure 5B). To gain primary molecular insight, we examined downstream activation upon

BCR engagement in baseline or relapse setting in samples obtained from a single patient who developed ibrutinib resistance (patient #2 in Table 1). Although stronger induction of *p*-ERK is ineffectively suppressed by ibrutinib in the relapse sample, dasatinib, GS-9973, or R406 successfully reversed downstream signaling activation (Figure 5C). Together, our results verify both SYK and LYN as therapeutic targets in patients with acquired PLC γ 2 hypermorphic mutations.



Figure 6. The diagram illustrates PLCG2^{R665W}-mediated ibrutinib resistance. In treatment naïve CLL, proximal BCR signaling triggers downstream BTK activation. PLC₇2 is consequently activated in a BTK-dependent manner. Targeting BTK by ibrutinib can abrogate PLC₇2-initiated downstream survival signal (left panel); in contrast, mutant PLC₇2 (R665W) can be activated via SYK or LYN, bypassing BTK dependency in the resistant CLL, thereby propagating downstream survival signals despite ibrutinib treatment (right panel). PKC, protein kinase C.

Discussion

Although microenvironment-derived CLL survival and proliferation stimuli are multifactorial, the BCR plays a critical role in driving disease progression; hence, ibrutinib has demonstrated exceptional clinical results in CLL. Nonetheless, relapses have been observed and understanding the molecular pathways that confer ibrutinib resistance is of the utmost priority in determining the treatment scheme in these patients. Given the predominant effect of ibrutinib in suppressing BCR signaling, the resistant mechanisms are speculated to emerge within this signaling pathway. In this study, extended from our previous report, genetic aberrations at PLCG2 are further elucidated in relapsed patients. Our data thoroughly characterize the competency of the R665W mutation to trigger downstream BCR signaling independent of BTK upon anti-IgM stimulation and explains why these patients are resistant to ibrutinib. We show here, that the proximal effectors SYK and LYN collaborate to activate mutated PLCy2 regardless of BTK activation. Moreover, pharmacologically targeting either SYK or LYN can overcome persistent survival signaling (Figure 6). Together, these data augment the understanding of mutated PLCy2-mediated ibrutinib resistance, and suggest alternative approaches to overcome the genetic abnormalities.

Similar to drug resistances that result from hampered affinity, mutations in BTK have been recognized in patients who relapsed on ibrutinib. These findings further support the prominent role of BCRmediated CLL progression. Intriguingly, BTK remains normal in a subset of relapsed patients with *PLCG2*^{R665W}, suggesting that *PLCG2*^{R665W} is sufficient to induce ibrutinib resistance. Remarkably, the aberration in PLC γ 2 appears to be somatically acquired only after long-term ibrutinib treatment, suggesting that this clone evolved under continuous pressure from this irreversible agent. Indeed, although subgroups of CLL drive toward anergy, differential capacity of BCR signaling has been demonstrated to correlate with surface IgM expression.²⁵ Although comparable sIgM was detected in DT40 expressing PLC γ 2^{R665W}, CLL with this hypermorphic aberration may enhance the survival advantage and gain access to tissue environment.²⁵ PLC γ 2 activity is correlated with tyrosine

phosphorylation at 753, 759, 1197, and 1217.^{26,27} On a molecular level, the phospholipase activity is repressed by the auto-inhibitory region encompassed within X- and Y-box domains. Recent studies indicate the crucial function of carboxyl-terminal SH2 in the regulatory element in suppressing PLCy2 activity.²⁶ The inherited S707Y mutation at this region has been reported to confer a hypermorphic effect and leads to an auto-inflammatory response.²⁴ Consistent with this notion, R665W residing within this region appears to elicit an analogous mechanism to impose enzymatic activity via releasing the catalytic domain of PLC γ 2 through a peptide conformation change. Nevertheless, direct phosphorylation sites triggered by BTK, such as Tyr1217, remains functionally inhibited in the presence of ibrutinib despite hyperactive downstream signaling events induced by mutated PLC γ 2 (Figure 2A), affirming the activation of mutant PLC γ 2 is discrete from BTK activity. Although previous studies have confirmed the indispensable role of BTK for PLC γ 2 activation,¹⁴ mutated PLC γ 2 may shift the functional dependency to other proximal kinases. Moreover, PLCy2 activation also requires membrane docking; a process recruited by BLNK (SLP-65),²⁸ suggesting the possibility that mutated PLC γ 2 may be retained at the plasma membrane through positive modulation by BLNK. In regard to proximal kinases that initiate PLC γ 2 activation, our data shows that SYK and LYN are essential for the activation of mutated PLC₂. Considering membrane localization requires interactions between Pleckstrin homology domain at PLCy2 and phosphatidylinositol lipids within the plasma membrane,^{29,30} PI3K inhibitors were also evaluated as potential alternatives. Nevertheless, our data indicates neither idelalisib nor IPI-145, inhibitors of PI3K p1108 and/or p110 γ subunits in clinical use, could abrogate PLCG2^{R665W}mediated downstream signaling despite AKT inhibition (supplemental Figure 5).

R665W aberration at PLC $\gamma 2$ is in close proximity to identified phosphorylation sites, Tyr753 and Tyr 759, raising the possibility that the mutation may lead to conformational change of PLC $\gamma 2$, amplifying its catalytic function upon stimulation regardless of the phosphorylation status at these tyrosine residues. However, it is also unknown whether the hypermorphic mutation could acquire higher affinity to SYK, LYN, or BLNK, leading to sustained activity after stimulation.

It is important to note that at most 45% of the variant frequencies are at R665W; however, the frequency of this aberration is variable when measured in peripheral blood samples from relapsed CLL patients, denoting that resistant clones bearing PLCG2^{R665W} might enrich within bone marrow or lymphoid organs. Likewise, advanced sequencing technologies are in demand to certify if the aberration existed with extremely low frequency at baseline. Alternatively, cooperative scenarios leading to resistance may also contribute to relapse settings. Conforming to this, *BTK* or other *PLCG2* mutations were concurrently observed in relapsed patients with R665W (data not shown), suggesting additional aberrations at this signaling axis may synergistically contribute to CLL progression. Further studies will be required to fully elucidate this. Finally, despite the elusive origins of these abnormalities, work is underway to scrutinize the functional roles of diverse aberrations in PLC γ 2.

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Authorship

Contribution: T.-M.L., J.A.W., and Y.Z. designed the research, performed experiments, analyzed data, generated figures, and wrote the manuscript; E.S. and S.D. were involved in planning the research

References

- Gaidano G, Foà R, Dalla-Favera R. Molecular pathogenesis of chronic lymphocytic leukemia. *J Clin Invest.* 2012;122(10):3432-3438.
- Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med. 2005;352(8): 804-815.
- Herishanu Y, Pérez-Galán P, Liu D, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood.* 2011;117(2):563-574.
- Stevenson FK, Caligaris-Cappio F. Chronic lymphocytic leukemia: revelations from the B-cell receptor. *Blood.* 2004;103(12):4389-4395.
- Hallek M, Fischer K, Fingerle-Rowson G, et al; International Group of Investigators; German Chronic Lymphocytic Leukaemia Study Group. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet.* 2010;376(9747):1164-1174.
- Tam CS, O'Brien S, Wierda W, et al. Long-term results of the fludarabine, cyclophosphamide, and rituximab regimen as initial therapy of chronic lymphocytic leukemia. *Blood.* 2008;112(4): 975–980.
- Burger JA. Nurture versus nature: the microenvironment in chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program.* 2011;2011:96-103.
- Fais F, Ghiotto F, Hashimoto S, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J Clin Invest.* 1998;102(8):1515-1525.
- Damle RN, Wasil T, Fais F, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood.* 1999;94(6):1840-1847.
- Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood.* 1999;94(6): 1848-1854.

- Packham G, Krysov S, Allen A, et al. The outcome of B-cell receptor signaling in chronic lymphocytic leukemia: proliferation or anergy. *Haematologica*. 2014;99(7):1138-1148.
- 12. Woyach JA, Johnson AJ, Byrd JC. The B-cell receptor signaling pathway as a therapeutic target in CLL. *Blood.* 2012;120(6):1175-1184.
- Woyach JA, Furman RR, Liu TM, et al. Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. *N Engl J Med.* 2014; 370(24):2286-2294.
- Takata M, Kurosaki T. A role for Bruton's tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-gamma 2. J Exp Med. 1996;184(1):31-40.
- Reth M, Wienands J. Initiation and processing of signals from the B cell antigen receptor. *Annu Rev Immunol.* 1997;15:453-479.
- Kurosaki T, Maeda A, Ishiai M, Hashimoto A, Inabe K, Takata M. Regulation of the phospholipase C-gamma2 pathway in B cells. *Immunol Rev.* 2000;176:19-29.
- Wang D, Feng J, Wen R, et al. Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. *Immunity*. 2000;13(1): 25-35.
- Takata M, Sabe H, Hata A, et al. Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca2+ mobilization through distinct pathways. *EMBO J.* 1994;13(6):1341-1349.
- Kurosaki T, Tsukada S. BLNK: connecting Syk and Btk to calcium signals. *Immunity*. 2000; 12(1):1-5.
- Mócsai A, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat Rev Immunol.* 2010; 10(6):387-402.
- Pleiman CM, Clark MR, Gauen LK, et al. Mapping of sites on the Src family protein tyrosine kinases p55blk, p59fyn, and p56lyn which interact with the effector molecules phospholipase C-gamma 2, microtubule-associated protein kinase, GTPaseactivating protein, and phosphatidylinositol 3-kinase. *Mol Cell Biol.* 1993;13(9):5877-5887.

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Correspondence: Amy J. Johnson, The Ohio State University, OSUCCC Bldg, Room 455C, 410 W. 12th Ave, Columbus, OH 43210; e-mail: amy.johnson@osumc.edu.

- Maddocks KJ, Ruppert AS, Lozanski G, et al. Etiology of ibrutinib therapy discontinuation and outcomes in patients with chronic lymphocytic leukemia. JAMA Oncol. 2015;1(1):80-87.
- Ombrello MJ, Remmers EF, Sun G, et al. Cold urticaria, immunodeficiency, and autoimmunity related to PLCG2 deletions. *N Engl J Med.* 2012; 366(4):330-338.
- Zhou Q, Lee GS, Brady J, et al. A hypermorphic missense mutation in PLCG2, encoding phospholipase Cγ2, causes a dominantly inherited autoinflammatory disease with immunodeficiency. *Am J Hum Genet*. 2012;91(4): 713-720.
- Coelho V, Krysov S, Steele A, et al. Identification in CLL of circulating intraclonal subgroups with varying B-cell receptor expression and function. *Blood.* 2013;122(15):2664-2672.
- Watanabe D, Hashimoto S, Ishiai M, et al. Four tyrosine residues in phospholipase C-gamma 2, identified as Btk-dependent phosphorylation sites, are required for B cell antigen receptor-coupled calcium signaling. *J Biol Chem.* 2001;276(42): 38595-38601.
- Ozdener F, Dangelmaier C, Ashby B, Kunapuli SP, Daniel JL. Activation of phospholipase Cgamma2 by tyrosine phosphorylation. *Mol Pharmacol.* 2002;62(3):672-679.
- Ishiai M, Kurosaki M, Pappu R, et al. BLNK required for coupling Syk to PLC gamma 2 and Rac1-JNK in B cells. *Immunity*. 1999;10(1): 117-125.
- Falasca M, Logan SK, Lehto VP, Baccante G, Lemmon MA, Schlessinger J. Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. *EMBO* J. 1998;17(2):414-422.
- Bae YS, Cantley LG, Chen CS, Kim SR, Kwon KS, Rhee SG. Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem.* 1998;273(8): 4465-4469.