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Acquired mutations associated with ibrutinib resistance in Waldenström macroglobulinemia

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Key Points

- BTK^{Cys481} mutations, including multiple mutated variants within individual patients are common in ibrutinib-progressing WM patients.
- BTK^{Cys481} mutations were associated with mutated CXCR4 in WM patients progressing on ibrutinib.

Ibrutinib produces high response rates and durable remissions in Waldenström macroglobulinemia (WM) that are impacted by MYD88 and $CXCR4^{WHIM}$ mutations. Disease progression can develop on ibrutinib, although the molecular basis remains to be clarified. We sequenced sorted $CD19^{+}$ lymphoplasmacytic cells from 6 WM patients who progressed after achieving major responses on ibrutinib using Sanger, TA cloning and sequencing, and highly sensitive and allele-specific polymerase chain reaction (AS-PCR) assays that we developed for Bruton tyrosine kinase (*BTK*) mutations. AS-PCR assays were used to screen patients with and without progressive disease on ibrutinib, and ibrutinib-naïve disease. Targeted next-generation sequencing was used to validate AS-PCR findings, assess for other *BTK* mutations, and other targets in B-cell receptor and MYD88 signaling. Among the 6 progressing patients, 3 had BTK^{Cys481} variants that included $BTK^{Cys481Ser(c.1635G>C\ and\ c.1634T>A)}$ and $BTK^{Cys481Arg(c.1634T>C)}$. Two of these patients had multiple *BTK* mutations. Screening of 38 additional patients on ibrutinib without clinical progression identified BTK^{Cys481} mutations in 2 (5.1%) individuals, both of whom subsequently progressed. BTK^{Cys481} mutations were not detected in baseline samples or in 100 ibrutinib-naive WM patients. Using mutated *MYD88* as a tumor marker, BTK^{Cys481} mutations were subclonal, with a highly variable clonal distribution. Targeted deep-sequencing confirmed AS-PCR findings, and identified an additional $BTK^{Cys481Tyr(c.1634G>A)}$ mutation in the 2 patients with multiple other BTK^{Cys481} mutations, as well as $CARD11^{Leu878Phe(c.2632C>T)}$ and $PLC\gamma2^{Tyr495His(c.1483T>C)}$ mutations. Four of the 5 patients with BTK^{C481} variants were CXCR4 mutated. BTK^{Cys481} mutations are common in WM patients with clinical progression on ibrutinib, and are associated with mutated *CXCR4*. (*Blood*. 2017;129(18):2519-2525)

Introduction

Activating somatic mutations in *MYD88* and the C-terminal domain of *CXCR4* (warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis-like) are present in ~90% to 95% and 30% to 40% of Waldenström macroglobulinemia (WM) patients, respectively.[1-4] *MYD88* mutations trigger pro-survival nuclear factor-κB signaling through Bruton tyrosine kinase (BTK), which is a target of ibrutinib. *CXCR4* mutations are similar to those found in the germ line warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis syndrome, promote AKT and extracellular signal-regulated kinase 1/2 activation, and are associated with both in vitro and clinical drug resistance to ibrutinib.[4-7] These findings prompted clinical investigation of ibrutinib in previously treated WM patients who showed high levels of response activity and durable responses, and supported the regulatory approval of ibrutinib in WM in the United States and Europe.[8,9] An important revelation in this study was the role of *MYD88* and *CXCR4* mutation status as determinants of primary response in WM.[10] Patients who lacked *MYD88* mutations (ie, were wild-type [WT] for *MYD88*) had no major responses, whereas those with *MYD88* mutations who were *CXCR4* mutated had fewer major responses vs those WT for *CXCR4* (62% vs 92%). Furthermore, major responses were also delayed by 6 months or more for *CXCR4*-mutated individuals. High response rates with durable activity were also observed in a multicenter study that administered ibrutinib to heavily pre-treated, rituximab refractory WM patients.[11] Patients with *CXCR4* mutations also showed delayed responses, and the 1 patient with WT *MYD88* included in this study showed no response to ibrutinib. Despite the highly active nature of ibrutinib in WM, clinical progression occurs and mechanistic insights are lacking in WM. Among chronic lymphocytic leukemia (CLL) patients who progressed on ibrutinib, mutations in the ibrutinib binding (BTK^{Cys481}), gatekeeper (BTK^{Thr474}), and SH2 non-kinase (BTK^{Thr316}) domains of BTK have been reported.[12-15] Loss of ibrutinib binding due to BTK^{Cys481} mutations permits downstream survival signaling that includes *PLCγ2* and *CARD11*. Activating mutations in *PLCγ2* and *CARD11* have also been

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identified in CLL patients progressing on ibrutinib, whereas BTK^{Cys481} and *CARD11* mutations have also been identified in mantle cell lymphoma (MCL) patients who progressed on ibrutinib.[14-17]

We therefore examined sorted lymphoplasmacytic cells from WM patients with progressive disease on ibrutinib, on ibrutinib without clinical progression at time of bone marrow (BM) sampling, as well as untreated and previously treated ibrutinib-naïve patients, for mutations known to be associated with ibrutinib progression. For these efforts, we used Sanger, as well as cloning and sequencing studies to identify BTK^{Cys481} mutations associated with clinical progression in WM, and developed highly sensitive and allele-specific polymerase chain reaction (AS-PCR) assays for their detection to perform screening. Targeted next-generation sequencing (NGS) was also used to validate AS-PCR findings, and to assess for other *BTK* mutations, as well as *MYD88*, *CXCR4*, and other select targets in B-cell receptor (BCR) and MYD88 signaling (*PLCγ2*, *CARD11*, *HCK*, and *LYN*).

Patients and methods

Patient samples and methods

We identified 6 WM patients who had a major response and subsequently progressed on ibrutinib. All 6 of these patients had relapsed disease, and were symptomatic at time of ibrutinib initiation. Their clinical and laboratory characteristics are shown in Table 1. The median time to progression on ibrutinib for these patients was 16.3 (range, 7.7-37.1) months. We used Sanger, as well as cloning and sequencing studies to screen these patients for *BTK* mutations. We subsequently developed and validated highly sensitive and specific nested AS-PCR assays for 3 *BTK* mutations that we identified, and screened 38 patients on ibrutinib without clinical progression at the time of repeat BM sampling, as well as 100 (50 untreated, 50 previously treated) ibrutinib-naïve patients. Targeted, deep NGS was used to confirm findings, to assess MYD88 and CXCR4 mutation status, and assess for other mutations in *BTK*, as well as *MYD88*, *CXCR4*, and other targets in BCR and MYD88 signaling. $CD19^+$ cells from BM aspirates were isolated, and DNA was extracted as previously described and used for sample analysis.[18,19] Subject participation was approved by the Harvard Cancer Center/Dana-Farber Cancer Institute's Institutional Review Board, and all participants provided written consent for use of their samples.

Sanger sequencing and cloning of BTK^{Cys481} mutants

A 382 bp fragment covering the BTK^{Cys481} was amplified by PCR. The forward and reverse PCR primers were 5′-TGAGAAGCTGGTGCAGTTGTATG-3′ and 5′-CTGGAGATATTTGATGGGCTCAG-3′, respectively. The amplified PCR products were isolated by QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and sequenced using forward and reverse PCR primers. PCR products were cloned into the TA cloning vector, and selected colonies sequenced using the M13 primers (Genewiz, South Plainfield, NJ).

Development of quantitative AS-PCR assays for *BTK* mutations at Cys481

Highly sensitive nested-PCR assays were developed to detect known BTK^{Cys481} mutations that included $BTK^{Cys481Ser(c.1635G>C\ and\ c.1634T>A)}$ and $BTK^{Cys481Arg(c.1634T>C)}$.

The primers are listed in supplemental Table 1 on the *Blood* Web site, and the assay conditions are presented in the supplemental Appendix. The amplification plots, dissociation curves, and standard curves for $BTK^{Cys481Arg(c.1634T>C)}$, $BTK^{Cys481Ser(c.1634T>A)}$, and $BTK^{Cys481Ser(c.1635G>C)}$ assays are presented in supplemental Figure 1.

Estimation of WM cell fraction that expressed BTK^{Cys481} mutations

To estimate the proportion of WM cells that expressed BTK^{Cys481} mutations, $MYD88^{L265P}$ was assumed as a tumor marker. The fraction of cells expressive of BTK^{Cys481} and $MYD88^{L265P}$ mutations was determined by ΔC_T and standard curves. The ratio of cells expressing $BTK^{Cys481}/MYD88^{L265P}$ was calculated. Copy number variants for the $MYD88^{L265P}$ locus were also determined by a TaqMan assay.[20] Using targeted deep sequencing analysis, the ratio of mutated $BTK^{Cys481}/MYD88$ allele frequency was determined.

Targeted deep sequencing

Targeted deep NGS was performed for 8 patients that included 6 patients with ibrutinib progression, and 2 others on active ibrutinib without clinical progression at the time of sampling who were positive for *BTK* mutations by AS-PCR. Tumor samples from the 8 patients, along with germ line (CD19-depleted peripheral blood mononuclear cells) samples available for 6 of these patients were sequenced. The library was generated using HaloPlexHS 1-500kb (Agilent Technologies, Santa Clara, CA), and sequencing data were generated from MiSeq paired-end sequences and aligned to HG19/GRCh37 ensemble genome reference using SureCall (http://www.agilent.com/search/?Ntt=surecall).[21] Molecular bar coding was used to identify reads, and reads supporting each call were calculated using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA). The median number of reads per patient was 774 (range, 372-1895). The genes included in the targeted deep sequencing included *BTK*, *PLCγ2*, *CARD11*, *LYN*, *HCK*, *MYD88*, and *CXCR4*.

Results

Sanger, cloning, and sequencing results for WM patients who progressed on ibrutinib

Six WM patients who progressed on active ibrutinib therapy were sequenced for BTK^{Cys481} mutations. Of the 6 patients, 5 had a $MYD88^{L265P}$ and 1 had a $MYD88^{S243N}$ mutation. Three of these 6 patients (50%) also had *CXCR4* mutations. By Sanger sequencing, a $BTK^{Cys481Arg(c.1634T>C)}$ mutation was identified in patient WM2, whereas a $BTK^{Cys481Ser(c.1634T>A)}$ was found in patient WM3 (Figure 1A). Cloning and sequencing analysis confirmed the presence of these mutations, and identified additional BTK^{Cys481} mutations in both patients as follows: 17/107 (15.9%), 21/107 (19.6%), and 7/107 (6.5%) clones expressed $BTK^{Cys481Arg(c.1634T>C)}$, $BTK^{Cys481Ser(c.1634T>A)}$, and $BTK^{Cys481Ser(c.1635G>C)}$, respectively, for patient WM2; whereas 2/119 (1.7%), 46/119 (38.7%), and 8/119 (6.7%) clones expressed $BTK^{Cys481Arg(c.1634T>C)}$, $BTK^{Cys481Ser(c.1634T>A)}$, and $BTK^{Cys481Ser(c.1635G>C)}$, respectively, for patient WM3. Baseline samples were available for 5 of the 6 progressing patients (all except WM2). No BTK mutations were detected by Sanger, and cloning and sequencing studies, in these samples. Representative tracings for the cloning and sequencing studies are shown in Figure 1B.

Detection of BTK^{Cys481} mutations in WM patients who progressed on ibrutinib using nested AS-PCR

The findings from the cloning and sequencing analyses encouraged us to develop more sensitive methods to evaluate for ibrutinib-resistant clones. To ensure high sensitivity and reliability, we developed nested AS-PCR assays for $BTK^{Cys481Arg(c.1634T>C)}$, $BTK^{Cys481Ser(c.1634T>A)}$, and $BTK^{Cys481Ser(c.1635G>C)}$. The AS-PCR assays developed for $BTK^{Cys481Ser(c.1635G>C)}$ and $BTK^{Cys481Ser(c.1634T>A)}$ detected these mutations at a dilution of 0.1%, and for $BTK^{Cys481Arg(c.1634T>C)}$ at a dilution of 0.8% with ≥2-cycle difference from the WT DNA background. The details for the development of these assays are presented in the supplemental Appendix.

We then applied the nested AS-PCR assays to the 6 WM patients who progressed on ibrutinib. The samples from baseline and the time of disease progression were analyzed in parallel. We identified by nested

Table 1. Baseline clinical and laboratory characteristics of WM patients who progressed on ibrutinib

Baseline characteristics	WM1	WM2	WM3	WM4	WM5	WM6
Age (y)	77	92	61	66	78	73
Sex	Male	Male	Male	Male	Male	Male
Serum IgM (mg/dL)	4130	921	3630	2490	1300	5790
Serum IgM M-spike (g/dL)	2.21	0.86	2.09	1.41	0.98	3.62
Hb (g/dL)	10.3	8.0	12.3	9.1	8.9	10.8
Serum β_2-microglobulin (mg/L)	4.5	N/A	2.5	5.6	14.2	3.9
BM involvement (%)	30	N/A	70	40	5	30
Prior therapies	Fludarabine, R, CPR, bendamustine-R, bortezomib/dex, tositumomab	R	R, ofatumumab, C, pentostatin	R	Cladribine, C, R, IFN-α, bendamustine, bortezomib/dex	R, chlorambucil, bendamustine
Time on ibrutinib (mo)	9.6	7.7	37.1	36.4	9.1	23.0
Best response to ibrutinib	PR	PR	PR	VGPR	VGPR	PR
Events supporting progressive disease from best response	BM 30%→70% IgM M-spike 0.55→1.12 Hb 11.8→10.5	IgM M-spike NQ→0.55 Hb 9.5→7.3	BM 20%→90% Hb 13.4→10.7	Splenic enlargement Hb 10.6→7.8 New pleural effusion	BM 5%→60% IgM M-spike 0.23→0.81 Hb 9.6→8.4	BM 15→80% IgM 2647→3970 Hb 15.1→7.7
MYD88 status	L265P	L265P	L265P	S243N	L265P	L265P
CXCR4 status	WT	S339fs	S338X	WT	WT	S338fs

C, cyclophosphamide; dex, dexamethasone; fs, denotes frameshift mutation present at this amino acid site; Hb, hemoglobin; IFN, interferon; IgM, immunoglobulin M; N/A, not available; NQ, faint, not quantifiable; P, prednisone; PR, partial response; R, rituximab; VGPR, very good partial response.

AS-PCR assays the three BTK^{Cys481} mutations, which we found by Sanger, and cloning and sequencing analyses, in patients WM2 and WM3. In addition, a $BTK^{Cys481Ser(c.1635G>C)}$ mutation was also identified in patient WM6 that was not detected by Sanger sequencing. Thus, the nested AS-PCR assays were able to identify BTK^{Cys481} mutations in 3 of 6 WM patients who progressed on ibrutinib that included the 2 patients with multiple BTK^{Cys481} mutations. All 3 of these patients also carried $MYD88^{L265P}$ and *CXCR4* mutations. Baseline samples were available for 5 of the 6 progressing patients (all except WM2). No BTK mutations were detected by AS-PCR studies in these samples.

To better understand the proportion of tumor cells expressing BTK^{Cys481} mutations in these patients, $MYD88^{L265P}$ was assumed as a tumor marker and the ratio of cells expressing BTK^{Cys481} to $MYD88^{L265P}$ was calculated. No copy number variants in the $MYD88^{L265P}$ locus were detected by TaqMan assays in any of the patients. The fraction of cells expressing $BTK^{Cys481Arg(c.1634T>C)}$, $BTK^{Cys481Ser(c.1634T>A)}$, and $BTK^{Cys481Ser(c.1635G>C)}$ relative to $MYD88^{L265P}$ was 32.3%, 12.9%, and 4.2%, respectively, for patient WM2; and 1.2%, 28.0%, and 2.5%, respectively, for patient WM3. Therefore, the combined fraction of cells expressing the BTK^{Cys481} mutations relative to $MYD88^{L265P}$ were 49.4% and 31.7% for patients WM2 and WM3, respectively. In patient WM6, the fraction of cells expressing $BTK^{Cys481Ser(c.1635G>C)}$ relative to $MYD88^{L265P}$ was 3.8%.

Serial samples were only available from patient WM3 to permit multiple time point longitudinal analysis for BTK^{Cys481} mutations by nested AS-PCR assays. This patient had cryoglobulinemia that resulted in erratic IgM readings. Serial BM and Hb levels were therefore tracked. At baseline, the patient had fatigue with an Hb level of 12.3 g/dL and BM tumor involvement of 80%. At month 12, the patient reported a good energy level with an Hb level of 13.4 g/dL and a repeat BM biopsy showed 50% involvement. At month 24, the patient continued to describe a good energy level with an Hb level of 13.3 g/dL, and BM biopsy showed 20% tumor infiltration. By month 36, the patient was again complaining of fatigue, with a decrease in Hb level to 9.3 g/dL, and a repeat BM biopsy showed 90% tumor infiltration.

Coincident with the above time points, no BTK^{Cys481} mutations were detected by AS-PCR assays at baseline or at month 12. At month 24, $BTK^{Cys481Ser(c.1634T>A)}$ and $BTK^{Cys481Ser(c.1635G>C)}$ mutations were first detected, and constituted 0.71% and 0.19% of the total $MYD88^{L265P}$ clone. By month 36, the $BTK^{Cys481Ser(c.1634T>A)}$ and $BTK^{Cys481Ser(c.1635G>C)}$ mutations were markedly higher at 26.1% and 3.62% of the total $MYD88^{L265P}$ clone. In addition, the $BTK^{Cys481Arg(c.1634T>C)}$ clone was also first detectable at month 36 for this patient, and made up 2.54% of the total $MYD88^{L265P}$ clone.

Screening for BTK^{Cys481} mutations by nested AS-PCR assays in patients on active ibrutinib therapy without clinical progression

We next applied the nested AS-PCR assays to samples from 38 patients on active ibrutinib therapy without clinical progression. The median time on ibrutinib for these patients at last BM sampling was 31.9 (range, 3.9-41.3) months, and the median time on ibrutinib was 45.1 (range, 3.9-not reached) months. Among these patients, 2 (5.1%) expressed $BTK^{Cys481Ser(c.1635G>C)}$ variants in samples taken after 24 months on ibrutinib. Baseline samples were available for both of these patients, and no BTK mutations were detected by AS-PCR assays. The estimated fraction of cells expressing $BTK^{Cys481Ser(c.1635G>C)}$ relative to $MYD88^{L265P}$ was 2.0% for patient WM7, and 1.0% for patient WM8 at last BM sampling. A *CXCR4* mutation was also detected in patient WM7 at time of last BM sampling. Subsequently, 6 patients had progressive disease, including 3 with and 3 without systemic progression (2 amyloidosis and 1 Bing-Neel syndrome). Included among the 3 patients with systemic progression were patients WM7 and WM8, both of whom had refractory disease with 85% and 90% disease involvement, respectively, at time of ibrutinib initiation. The time on ibrutinib for these patients was 21.3 and 21.4 months at time of BM sampling for BTK^{Cys481} variants. Both achieved a very good partial response, and progressed to 18.5 and 9.2 months following detection of BTK^{Cys481} variants.

Screening for BTK^{Cys481} mutations by nested AS-PCR assays in ibrutinib-naïve patients

We next applied the nested AS-PCR assays to samples from 50 untreated and 50 previously treated ibrutinib-naïve patients. No $BTK^{Cys481Arg(c.1634T>C)}$, $BTK^{Cys481Ser(c.1634T>A)}$, or $BTK^{Cys481Ser(c.1635G>C)}$ mutations were detected by the AS-PCR assays in any of these patients.

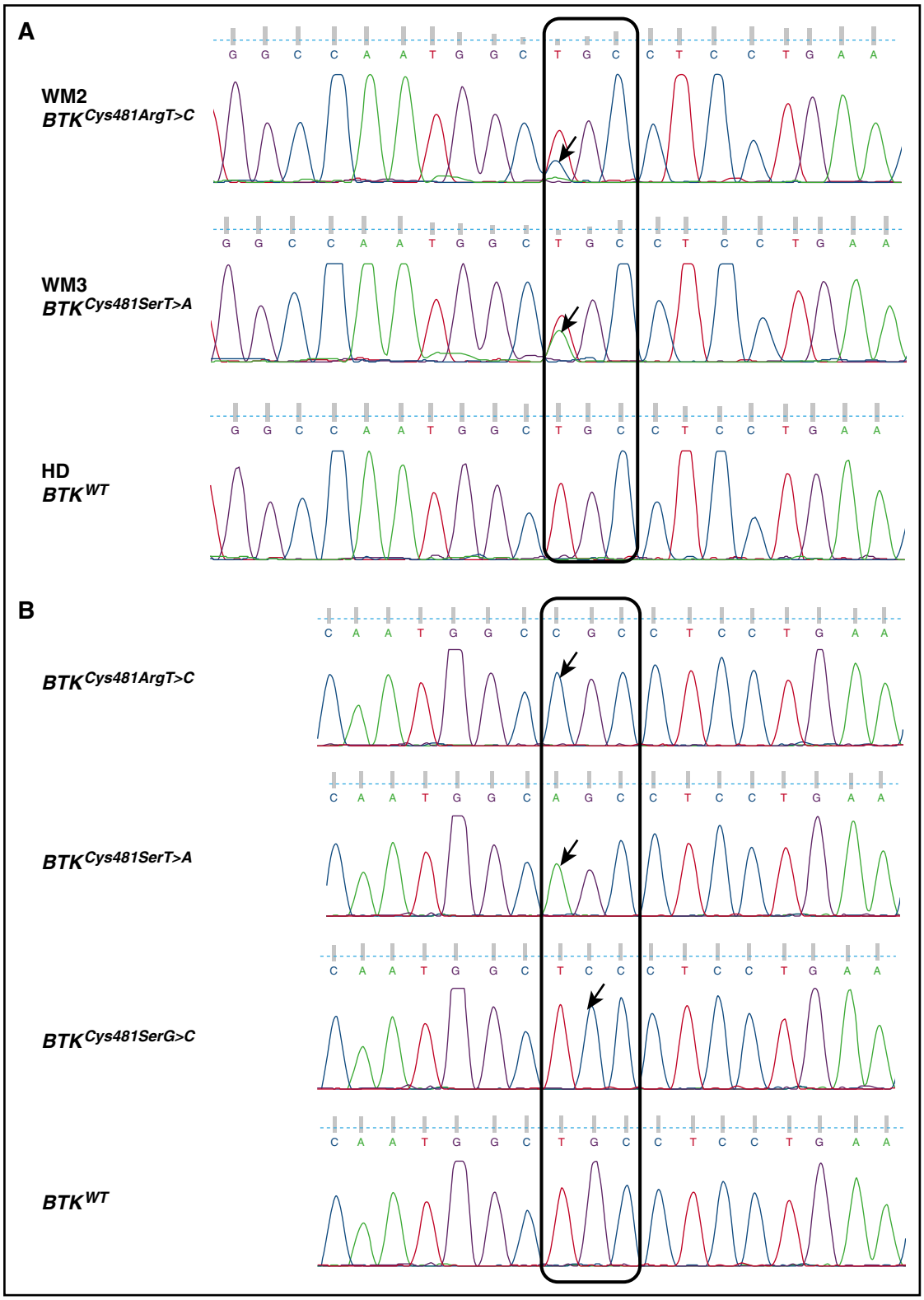

Figure 1. Representative Sanger sequencing traces for patients with BTK^{Cys481} variants. (A) Sanger sequencing traces on CD19-sorted BM cells showing $BTK^{Cys481Arg(c.1634T>C)}$ and $BTK^{Cys481Ser(c.1634T>A)}$ variants in patients WM2 and WM3, respectively. (B) Representative Sanger sequencing traces from cloning and sequencing studies show the presence of multiple BTK^{Cys481} variants in patient WM2. Cloning and sequencing analysis showed 17/107 (15.9%), 21/107 (19.6%), and 7/107 (6.5%) clones expressed $BTK^{Cys481Arg(c.1634T>C)}$, $BTK^{Cys481Ser(c.1634T>A)}$, and $BTK^{Cys481Ser(c.1635G>C)}$, respectively, for patient WM2; whereas 2/119 (1.7%), 46/119 (38.7%), and 8/119 (6.7%) clones expressed $BTK^{Cys481Arg(c.1634T>C)}$, $BTK^{Cys481Ser(c.1634T>A)}$, and $BTK^{Cys481Ser(c.1635G>C)}$, respectively, for patient WM3. Arrows denote nucleotide variants. HD, healthy donor.

Table 2. Targeted deep sequencing results for *BTK*, *PLCγ2*, and *CARD11* mutations in WM patients

Patient	*BTK* $^{Cys481Arg(T>C)}$	*BTK* $^{Cys481Ser(T>A)}$	*BTK* $^{Cys481Ser(G>C)}$	*BTK* $^{Cys481Tyr(G>A)}$	*PLCγ2* $^{Tyr495His(T>C)}$	*CARD11* $^{Leu878Phe(C>T)}$
WM1	ND	ND	ND	ND	ND	ND
WM2	32.4%	6.6%	5.8%	1.0%	ND	ND
WM3	0.3%	34.4%	6.5%	0.3%	ND	0.2%
WM4	ND	ND	ND	ND	ND	ND
WM5	ND	ND	ND	ND	ND	ND
WM6	ND	ND	10.3%	ND	11.9%	ND
WM7	ND	ND	1.5%	ND	ND	ND
WM8	ND	ND	0.7%	ND	ND	ND

Results are presented for 6 patients who progressed on ibrutinib (WM1-WM6), and for 2 patients (WM7 and WM8) who were positive for a BTK^{Cys481} variant from screening 38 patients on ibrutinib without clinical progression at time of sequencing.

ND, not detected.

Targeted deep NGS

To confirm the AS-PCR findings and screen for other potential mutations associated with secondary ibrutinib resistance, targeted deep sequencing for *MYD88*, *CXCR4*, *BTK*, *PLCγ2*, *CARD11*, *LYN*, and *HCK* was performed for the 6 patients who progressed on ibrutinib, as well as the 2 patients on ibrutinib who were detectable by AS-PCR for $BTK^{Cys481Ser(c.1635G>C)}$ mutation. For these 8 patients, 6 (WM1, 3, 4, 5, 7, and 8) had paired CD19-depleted peripheral blood mononuclear cells for germ line comparison. All BTK^{Cys481} mutations that were detected by the AS-PCR assays were confirmed by targeted deep sequencing (Table 2). The paired sample analysis confirmed the somatic nature of the BTK^{Cys481} mutations in all 3 patients for whom a germ line sample was available (WM3, 7, and 8). In addition, targeted deep sequencing detected an additional BTK^{Cys481} mutation, ie, $BTK^{Cys481Tyr(c.1634G>A)}$ in patients WM2 and WM3. Thus, patients WM2 and WM3 had four distinct BTK^{Cys481} mutations. The fraction of cells with BTK^{Cys481} mutations relative to $MYD88^{L265P}$ was estimated by the deep targeted sequencing reads. As shown in Table 2, the fraction of cells expressing $BTK^{Cys481Arg(c.1634T>C)}$, $BTK^{Cys481Ser(c.1634T>A)}$, $BTK^{Cys481Ser(c.1635G>C)}$, and $BTK^{Cys481Tyr(c.1634G>A)}$ relative to $MYD88^{L265P}$ was 32.4%, 6.6%, 5.8%, and 1.0%, respectively, for patient WM2; and 0.3%, 34.4%, 6.5%, and 0.3%, respectively, for patient WM3. In aggregate, the total fraction of cells expressing any type of BTK^{Cys481} mutation relative to $MYD88^{L265P}$ was 45.8% and 41.5% for patients WM2 and WM3, respectively.

In addition to BTK^{Cys481}, targeted deep sequencing identified a $PLC\gamma2^{Tyr495His(c.1483T>C)}$ variant in patient WM6 who had a $BTK^{Cys481Ser(c.1635G>C)}$ mutation (Table 2). The fraction of cells expressing $BTK^{Cys481Ser(c.1635G>C)}$ and $PLC\gamma2^{Tyr495His(c.1483T>C)}$ relative to $MYD88^{L265P}$ was 10.3% and 11.9%, respectively, in this patient. The somatic nature of $PLC\gamma2^{Tyr495His(c.1483T>C)}$ was inferred for this patient who lacked germ line tissue by its rarity in healthy donors, ie, found in only 1 Asian (n = 12 561), but no European (n = 36 590), African (n = 4881), or Latino (n = 5764) individuals (http://exac.broadinstitute.org).[22] Lastly, a novel mutation, ie, $CARD11^{Leu878Phe(c.2632C>T)}$ was identified in patient WM3 who had multiple BTK^{Cys481} variants by targeted deep sequencing (Table 2). The fraction of cells expressing this mutation relative to $MYD88^{L265P}$ was 0.2% (4/1895 reads), and was absent in the germ line sample for this patient.

Discussion

Despite high response rates and durable remissions in WM, disease progression can occur on active ibrutinib therapy. Understanding the molecular mechanism(s) responsible for acquired resistance to ibrutinib may improve treatment strategy, and potentially direct novel drug discovery. We therefore performed a targeted genomic analysis in WM patients who progressed on active ibrutinib therapy using multiple sequencing approaches. Akin to CLL and MCL, BTK^{Cys481} mutations were frequently identified in progressing WM patients, and accounted for half of the progression events.

An important distinction was the multitude of BTK^{Cys481} mutations that were identified within individual WM patients. Using mutated *MYD88* as a tumor marker, BTK^{Cys481} mutations appeared to be primarily subclonal, with a highly variable clonal distribution. Among the 3 progressing patients with BTK^{Cys481} mutations, 2 (WM2 and WM3) had four different BTK^{Cys481} mutations, with frequencies that ranged from 0.3% to 34.4% for each mutation. No gatekeeper BTK^{Thr474} or non-Cys481 *BTK* mutations were observed. Baseline samples were available for 5 of the 6 progressing patients (all but WM2), as well as the 2 patients on active ibrutinib (WM7 and WM8), and were analyzed by Sanger sequencing and AS-PCR assays. No BTK mutations were detected suggesting that these mutations were acquired, although they could have existed at such very low clonal frequencies as to not be detectable by our assays. Similar observations have also been made in CLL patients progressing on ibrutinib with acquired BTK mutations. The highly variable and predominantly subclonal nature of BTK^{Cys481} variants in patients progressing on ibrutinib has also been observed in CLL, raising the possibility that other genomic or epigenomic events may also be contributing to resistance. It also remains possible that although the *BTK*-mutated clone is subclonal, it could be exerting pro-growth and/or survival effects directly or indirectly through micro-environmental interactions on neighboring non–*BTK*-mutated clones.

The finding of *CXCR4* mutations in 4 of the 5 patients with BTK^{C481} variants may allude to underlying genomic instability. Similar observations have been made in CLL patients, wherein *BTK* mutations appear more common in 17p (p53)-deleted patients.[12] Although *TP53* mutations are rare in WM, and were not observed in any of the ibrutinib-resistant patients in this series by targeted NGS (data not shown), dysregulated *RAG1*, *RAG2*, and *ATM* expression are commonly observed in WM.[23] Further insights into the role of these dysregulated genes, as well as other genomic or epigenomic variants that contribute to the acquisition of BTK^{C481} variants in WM patients on ibrutinib are needed. The findings may also indicate an increased susceptibility of *CXCR4*-mutated patients to develop BTK^{Cys481}-related resistance to ibrutinib therapy. A frontline study of single-agent ibrutinib in WM patients with serial deep whole exome sequencing is now fully enrolled and may help validate these observations (#NCT02604511). *CXCR4* mutations are subclonal in most WM patients with a highly variable clonality within the WM clone, as defined by mutated MYD88.[20] We were unable to address in these studies whether the BTK^{Cys481}

variants occurred within CXCR4-mutated cells, and prospective studies utilizing sorted single tumor cell sequencing are needed to clarify this important point.

The emergence and expansion kinetics of the various BTK^{Cys481} variants in WM patients who developed resistance to ibrutinib was also of interest. Although the underlying genomic background within WM clones could have contributed to the emergence and expansion kinetics of the various BTK^{Cys481} variants, the amino acid substitution itself may also be critical. A recent study used site-directed mutagenesis to examine different amino acid substitutions at BTK^{Cys481}.[24] Substitutions of cysteine by serine and threonine, but not tyrosine, tryptophan, phenylalanine, glycine, or arginine permitted BTK phosphorylation, and triggered downstream *PLCγ2* activation in the presence of ibrutinib. $BTK^{Cys481Ser}$ were the predominant or sole BTK^{Cys481} variants generated by either T>A or G>C transversions at n.1634 in 4 of 5 WM patients, and are observed in most CLL and MCL patients carrying a BTK^{Cys481} variant. However, in our series, a $BTK^{Cys481Arg}$ constituted the predominant variant in 1 patient (WM3) with multiple BTK^{Cys481} clones including $BTK^{Cys481Ser}$, and was observed in another patient (WM2), whereas $BTK^{Cys481Tyr}$ variants were seen in 2 patients (WM2 and WM3). Both $BTK^{Cys481Arg}$ and $BTK^{Cys481Tyr}$ would not have been predicted as promoting ibrutinib resistance.[24] Further mechanistic insights are therefore needed to clarify whether an underlying genomic instability in WM patients may account for the multitude of observed BTK^{Cys481} variants, and their potential functionality in *MYD88*-mutated patients.

Akin to CLL, we also observed a putative PLCγ2 variant in 1 WM patient who also had a $BTK^{Cys481SerG>C}$ mutation (WM6).[12,16] The $PLC\gamma2^{Tyr495His}$ mutation observed in the WM patient has not been previously reported, and is located within the auto-inhibitory domain wherein PLCγ2 mutations have been identified in ibrutinib-resistant CLL or MCL patients.[25] In vitro transduction studies have eluded to circumvention of BTK signaling by a *PLCγ2* mutation found in the auto-inhibitory domain in ibrutinib-resistant patients, and the potential to abrogate aberrant *PLCγ2* signaling by agents that target the BCR members LYN or SYK.[25] We also observed a $CARD11^{Leu878Phe}$ mutation at a very low frequency in a patient with multiple $BTK^{Cys481Ser}$ variants (WM3). *CARD11* mutations in the coil-coiled domain were observed in *MYD88*-mutated activated B-cell subtype of diffuse large B-cell lymphoma patients, and are associated with in vitro, as well as primary clinical resistance to ibrutinib in activated B-cell diffuse large B-cell lymphoma and MCL.[26-28] The variant observed by us has not been previously described and resides outside the coil-coiled domain of *CARD11*. Functional studies are therefore needed to characterize this novel mutation and its potential contribution to ibrutinib resistance.

In conclusion, the findings of this study provide the first reported insights into the molecular mechanisms associated with ibrutinib resistance in WM, and highlight the emergence of multiple *BTK*-mutated clones, including non-$BTK^{Cys481Ser}$ clones, as well as novel *PLCγ2* and *CARD11* mutations within individual patients who progressed on active ibrutinib therapy.

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Authorship

Contribution: S.P.T., L.X., and Z.R.H. designed the study; C.J.P., K.M., J.G., T.D., M.L.P., R.A., J.J.C., R.R.F., and S.P.T. collected study samples and data; L.X., N.T., G.Y., J.G.C., X.L., M.D., and A.K. processed tumor samples and performed mutation analysis; L.X., Z.R.H., and S.P.T. analyzed the study data; and L.X. and S.P.T. wrote the manuscript.

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