Acquired mutations associated with ibrutinib resistance in Waldenström macroglobulinemia

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Key Points
- BTK\textsuperscript{Cys481} mutations, including multiple mutated variants within individual patients are common in ibrutinib-progressing WM patients.
- BTK\textsuperscript{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\textsuperscript{WHM} mutations. Disease progression can develop on ibrutinib, although the molecular basis remains to be clarified. We sequenced sorted CD19\textsuperscript{+} 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\textsuperscript{Cys481} variants that included BTK\textsuperscript{Cys481Ser(c.1635G>A)} and BTK\textsuperscript{Cys481Arg(c.1634T>C)} mutations. Two of these patients had multiple BTK mutations. Screening of 38 additional patients on ibrutinib without clinical progression identified BTK\textsuperscript{Cys481} mutations in 2 (5.1%) individuals, both of whom subsequently progressed. BTK\textsuperscript{Cys481} mutations were not detected in baseline samples or in 100 ibrutinib-naïve WM patients. Using mutated MYD88 as a tumor marker, BTK\textsuperscript{Cys481} mutations were subclonal, with a highly variable clonal distribution. Targeted deep-sequencing confirmed AS-PCR findings, and identified an additional BTK\textsuperscript{Cys481Tyr(c.1634G>A)} mutation in the 2 patients with multiple other BTK\textsuperscript{Cys481} mutations, as well as CARD11\textsuperscript{Leu878Phe(c.2632C>T)} and PLC\textsubscript{γ2}\textsuperscript{Tyr495His(c.1483T>C)} mutations. Four of the 5 patients with BTK\textsuperscript{Cys481} variants were CXCR4 mutated. BTK\textsuperscript{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 observations clarified the role of MYD88 and CXCR4 mutation status as determinants of primary response in WM.8 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\textsuperscript{Cys481}), gatekeeper (BTK\textsuperscript{Thr474}), and SH2 non-kinase (BTK\textsuperscript{Thr316}) domains of BTK have been reported.12-15 Loss of ibrutinib binding due to BTK\textsuperscript{Cys481} mutations permits downstream survival signaling that includes PLC\textsubscript{γ2} and CARD11. Activating mutations in PLC\textsubscript{γ2} and CARD11 have also been
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 ($PLCy2$, $CARD11$, $HCK$, 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′-TGAAGAAGCTGGTGCAGTGTAGT3′ 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 $BTK^{Cys481Serg.c.1633G>C}$ 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 $BTK^{Cys481Serg.c.1633G>C}$, $BTK^{Cys481Serg.c.1634T>A}$, and $BTK^{Cys481Serg.c.1635G>C}$ 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, $MYD88^{265P}$ was assumed as a tumor marker. The fraction of cells expressive of $BTK^{Cys481}$ and $MYD88^{265P}$ mutations was determined by $\Delta C_T$ and standard curves. The ratio of cells expressing $BTK^{Cys481}$/$MYD88^{265P}$ was calculated. Copy number variants for the $MYD88^{265P}$ 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 1.50kb (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 $BTK$, $PLCy2$, $CARD11$, $HCK$, $MYD88$, 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 $MYD88^{265P}$ and 1 had a $MYD88^{243N}$ 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 $BTK^{Cys481Serg.c.1634T>A}$ 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 $BTK^{Cys481Arg.c.1634T>C}$, $BTK^{Cys481Serg.c.1634T>A}$, and $BTK^{Cys481Serg.c.1635G>C}$, respectively, for patient WM2; whereas 2/119 (1.7%), 46/119 (38.7%), and 8/119 (6.7%) clones expressed $BTK^{Cys481Arg.c.1634T>C}$, $BTK^{Cys481Serg.c.1634T>A}$, and $BTK^{Cys481Serg.c.1635G>C}$, 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 $BTK^{Cys481Arg.c.1634T>C}$, $BTK^{Cys481Serg.c.1634T>A}$, and $BTK^{Cys481Serg.c.1635G>C}$. The AS-PCR assays developed for $BTK^{Cys481Serg.c.1635G>C}$ and $BTK^{Cys481Serg.c.1634T>A}$ 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
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 (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 in 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 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.

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

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>WM1</th>
<th>WM2</th>
<th>WM3</th>
<th>WM4</th>
<th>WM5</th>
<th>WM6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>77</td>
<td>92</td>
<td>61</td>
<td>66</td>
<td>78</td>
<td>73</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>Serum IgM (mg/dL)</td>
<td>4130</td>
<td>921</td>
<td>3630</td>
<td>2490</td>
<td>1300</td>
<td>5790</td>
</tr>
<tr>
<td>Serum IgM M-spike (g/dL)</td>
<td>2.21</td>
<td>0.86</td>
<td>2.09</td>
<td>1.41</td>
<td>0.98</td>
<td>3.62</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>10.3</td>
<td>8.0</td>
<td>12.3</td>
<td>9.1</td>
<td>8.9</td>
<td>10.8</td>
</tr>
<tr>
<td>Serum $\mu$-microglobulin (mg/L)</td>
<td>4.5</td>
<td>N/A</td>
<td>2.5</td>
<td>5.6</td>
<td>14.2</td>
<td>3.9</td>
</tr>
<tr>
<td>BM involvement (%)</td>
<td>30</td>
<td>N/A</td>
<td>70</td>
<td>40</td>
<td>5</td>
<td>30</td>
</tr>
</tbody>
</table>

| Time on ibrutinib (mo)   | 9.6 | 7.7 | 37.1| 36.4| 9.1 | 23.0|
| Best response to ibrutinib | PR | PR | PR | PR | VGPR | VGPR |
| Events supporting progressive disease | BM 30%–?70% IgM | IgM M-spike | BM 20%–90% | Splenic enlargement Hb | BM 5%–60% IgM | BM 15–80% |
| from best response | M-spike 0.55–1.12 | NQ–0.55 | Hb 13.4–10.7 | 10.6–7.8 New pleural effusion | M-spike 0.23–?0.81 | IgM 2647–3970 |
| 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, taint, not quantifiable; P, prednisone; PR, partial response; R, rituximab; VGPR, very good partial response.
Figure 1. Representative Sanger sequencing traces for patients with BTK{superscript}Cys481{var} variants. (A) Sanger sequencing traces on CD19-sorted BM cells showing BTK{superscript}Cys481Arg{superscript} variants in patients WM2 and WM3, respectively. (B) Representative Sanger sequencing traces from cloning and sequencing studies show the presence of multiple BTK{superscript}Cys481{var} 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{superscript}Cys481Arg{superscript}, BTK{superscript}Cys481Ser{superscript}, and BTK{superscript}Cys481SerG{superscript} variants, respectively, for patient WM2; whereas 2/119 (1.7%), 46/119 (38.7%), and 8/119 (6.7%) clones expressed BTK{superscript}Cys481Arg{superscript}, BTK{superscript}Cys481Ser{superscript}, and BTK{superscript}Cys481SerG{superscript} variants, 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

<table>
<thead>
<tr>
<th>Patient</th>
<th>BTK Cys481Arg(T&gt;C)</th>
<th>BTK Cys481Ser(T&gt;A)</th>
<th>BTK Cys481Ser(G&gt;C)</th>
<th>BTK Cys481Tyr(D&gt;A)</th>
<th>PLCγ2 Tyr495His(T&gt;C)</th>
<th>CARD11 Leu708Phe(C&gt;T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>WM2</td>
<td>32.4%</td>
<td>6.6%</td>
<td>5.8%</td>
<td>1.0%</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>WM3</td>
<td>0.3%</td>
<td>34.4%</td>
<td>6.5%</td>
<td>0.3%</td>
<td>ND</td>
<td>0.2%</td>
</tr>
<tr>
<td>WM4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>WM5</td>
<td>ND</td>
<td>ND</td>
<td>10.3%</td>
<td>ND</td>
<td>ND</td>
<td>11.9%</td>
</tr>
<tr>
<td>WM6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>WM7</td>
<td>ND</td>
<td>ND</td>
<td>1.5%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>WM8</td>
<td>ND</td>
<td>ND</td>
<td>0.7%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Results are presented for 6 patients who progressed on ibrutinib (WM1-WM6), and for 2 patients (WM7 and WM8) who were positive for a BTKCys481 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 BTKCys481 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 BTKCys481 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 BTKCys481 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 BTKCys481 mutation, ie, BTKCys481Tyr(Tyr, 1634G>A) in patients WM2 and WM3. Thus, patients WM2 and WM3 had four distinct BTKCys481 mutations. The fraction of cells with BTKCys481 mutations relative to MYD88 variant was estimated by the deep targeted sequencing reads. As shown in Table 2, the fraction of cells expressing BTKCys481Arg(Tyr, 1634G>C), BTKCys481Ser(Tyr, 1634G>A), BTKCys481Ser(Tyr, 1634G>C), and BTKCys481Tyr(Tyr, 1634G>A) relative to MYD88 variant was 32.4%, 6.6%, 5.8%, 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 BTKCys481 mutation relative to MYD88 variant was 45.8% and 41.5% for patients WM2 and WM3, respectively.

In addition to BTKCys481, targeted deep sequencing identified a PLCγ2Tyr495His(Tyr, 1483T>C) variant in patient WM6 who had a BTKCys481Arg(Tyr, 1635G>C) mutation (Table 2). The fraction of cells expressing BTKCys481Arg(Tyr, 1635G>C) and PLCγ2Tyr495His(Tyr, 1483T>C) relative to MYD88 variant was 10.3% and 11.9%, respectively, in this patient. The somatic nature of PLCγ2Tyr495His(Tyr, 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 Leu708Phe(Tyr, 2603C>T) was identified in patient WM3 who had multiple BTKCys481 variants by targeted deep sequencing (Table 2). The fraction of cells expressing this mutation relative to MYD88 variant 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, BTKCys481 mutations were frequently identified in progressing WM patients, and accounted for half of the progression events.

An important distinction was the multitude of BTKCys481 mutations that were identified within individual WM patients. Using mutated MYD88 as a tumor marker, BTKCys481 mutations appeared to be primarily subclonal, with a highly variable clonal distribution. Among the 3 progressing patients with BTKCys481 mutations, 2 (WM2 and WM3) had four different BTKCys481 mutations, with frequencies that ranged from 0.3% to 34.4% for each mutation. No gatekeeper BTKThr474 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 BTKCys481 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-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 BTKCys481 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 BTKCys481 variants in WM patients on ibrutinib are needed. The findings may also indicate an increased susceptibility of CXCR4-mutated patients to develop BTKCys481-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 BTKCys481
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 $\text{BTK}^{\text{Cys481}}$ 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 $\text{BTK}^{\text{Cys481}}$ variants, the amino acid substitution itself may also be critical. A recent study used site-directed mutagenesis to examine different amino acid substitutions at $\text{BTK}^{\text{Cys481}}$. 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. $\text{BTK}^{\text{Cys481Ser}}$ were the predominant or sole $\text{BTK}^{\text{Cys481}}$ variants generated by either $\text{G} > \text{A}$ or $\text{G} > \text{C}$ transversions at n.1634 in 4 of 5 WM patients, and are observed in most CLL and MCL patients carrying a $\text{BTK}^{\text{Cys481}}$ variant. However, in our series, a $\text{BTK}^{\text{Cys481Ser}}$ constituted the predominant variant in 1 patient (WM3) with multiple $\text{BTK}^{\text{Cys481}}$ clones including $\text{BTK}^{\text{Cys481Ser}}$, and was observed in another patient (WM2), whereas $\text{BTK}^{\text{Cys481Thr}}$ variants were seen in 2 patients (WM2 and WM3). Both $\text{BTK}^{\text{Cys481Arg}}$ and $\text{BTK}^{\text{Cys481Ser}}$ would not have been predicted as promoting ibrutinib resistance. Further mechanistic insights are therefore needed to clarify whether an underlying genomic instability in WM patients may account for the multitude of observed $\text{BTK}^{\text{Cys481}}$ 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 $\text{BTK}^{\text{Cys481Ser}}$ mutation (WM6). The PLCγ2$^{\text{Tyr495His}}$ 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. 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. We also observed a $\text{CARD11}^{\text{Tyr287Pro}}$ mutation at a very low frequency in a patient with multiple $\text{BTK}^{\text{Cys481Ser}}$ 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. 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 $\text{BTK}^{\text{Cys481}}$-mutated clones, including non-$\text{BTK}^{\text{Cys481Ser}}$ clones, as well as novel PLCγ2 and CARD11 mutations within individual patients who progressed on active ibrutinib therapy.

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Authorship

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References


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