Genomic evolution of ibrutinib-resistant clones in Waldenström macroglobulinemia

Cristina Jiménez,1 Gloria G. Chan,1 Lian Xu,1 Nickolas Tsakmaklis,1 Amanda Kofides,1 Maria G. Demos,1 Jiaji Chen,1 Xia Liu,1 Manit Munshi,1 Guang Yang,1 Jorge J. Castillo,1,2 Adrian Wiestner,3 Ramón García-Sanz,4 Steven P. Treon1,2 and Zachary R. Hunter1,2
1Bing Center for Waldenstrom’s Macroglobulinemia, Dana-Farber Cancer Institute, 2Harvard Medical School, Boston, MA, 3Hematology Branch, National Heart, Lung and Blood Institute, Bethesda, MD, USA and 4Servicio de Hematología, Hospital Universitario de Salamanca, Instituto de Investigación Biomédica de Salamanca, Centro de Investigación Biomédica en Red Cáncer, Salamanca, Spain

Received 28 October 2019; accepted for publication 6 December 2019
Correspondence: Zachary R. Hunter, Bing Center for Waldenström’s Macroglobulinemia, Dana-Farber Cancer Institute, Harvard Medical School, Mayer 544, 450 Brookline Avenue, Boston, MA 02215, USA.
E-mail: Zachary_Hunter@dfci.harvard.edu

Summary
Ibrutinib is highly active in Waldenström macroglobulinemia (WM) patients, but disease progression can occur due to acquired mutations in BTK, the target of ibrutinib, or PLCG2, the protein downstream of BTK. However, not all resistant patients harbour these alterations. We have performed a whole-exome sequencing study to identify alternative molecular mechanisms that can drive ibrutinib resistance. Our findings include deletions on chromosomes 6q, including homozygous deletions, and 8p, which encompass key regulators of BTK, MYD88/NF-κB, and apoptotic signalling. Moreover, we have identified recurring mutations in ubiquitin ligases, innate immune signalling, and TLR/MYD88 pathway regulators in ibrutinib-resistant WM patients.

Keywords: Waldenström macroglobulinemia, whole-exome sequencing, ibrutinib, resistance, genomic alterations.

Somatic mutations in MYD88 occur in 95–97% of patients with Waldenström Macroglobulinemia (WM) (Treon et al., 2012; Jiménez et al., 2013). Secondary events involving heterozygous loss of chromosome 6q21-25 (del6q) or C-terminal domain mutations in CXCR4 are common in WM, accounting for 40–50% and 30–40% of MYD88-mutated patients, respectively (Xu et al., 2016; Guerrera et al., 2018). MYD88 mutations are activating, and trigger constitutive NF-κB activation through BTK and IRAK1/IRAK4 (Yang et al., 2013). BTK inhibitors, including ibrutinib, acalabrutinib and zanubrutinib, are highly active in MYD88-mutated but not MYD88wild-type WM patients (Argyropoulos & Palomba, 2018). CXCR4 mutations are associated with a lower rate of major responses and shorter progression-free survival (Treon et al., 2019). Acquired mutations in BTK at the binding site of ibrutinib (BTKCys481), or its downstream mediator PLCG2, have been identified in half of WM patients progressing on ibrutinib (Xu et al., 2017) and lead to enhanced growth and survival through the re-activation of MAPK3 and MAPK1 (ERK1/2) signalling (Chen et al., 2018). Re-activation of ERK1/2 triggers cytokine release that can confer ibrutinib resistance to neighbouring BTKwild-type cells. Not all WM patients progressing on ibrutinib harbour these alterations. We therefore performed whole-exome sequencing (WES) to study the genomics evolution of ibrutinib-resistant WM.

Five previously treated WM patients who progressed while on ibrutinib were included in this study. Clinical characteristics of this cohort are presented in Table I. Ibrutinib was administered at 420 mg/day until disease progression.
Median time to progression was 23 months (range 9.1–37.1 months). Bone marrow (BM) samples were collected at baseline and at the time of clinical progression for three patients. For two patients, only samples at time of progression were available. CD19-selected WM cells were obtained from BM samples using magnetic-activated cell sorting (Miltenyi-Biotec, Auburn, CA, USA). CD19-depleted peripheral blood cells from all five patients were used as germline controls. The study was approved by the Dana-Farber/Harvard Cancer Centre Institutional Review Board, and all samples were collected following informed consent.

WES was performed using the Haloplex Human exome capture kit (Agilent Technologies, Santa Clara, CA, USA) and 150 bp paired-end sequencing. Data were analysed following the GATK Best Practice Guidelines (Broad Institute, Cambridge, MA, USA). Reads were aligned to GRCh37/ HG19 using Burrows–Wheeler Aligner (BWA). Small variants were analysed using Strelka (Illumina, Inc., San Diego, CA, USA) and MutSigCV was used to identify significantly mutated genes (Benjamini–Hochberg q-value ≤0.1). Variants found in the Exome Aggregation Consortium (ExAC) database with allele frequency >0.01% and variants with scaled phred Combined Annotation Dependent Depletion (CADD) score <20 were removed from the analysis. Copy number alterations (CNA) were called using Control-FREEC (Boeva-Lab, Institut Curie, Paris, France). Kolmogorov-Smirnov and Wilcoxon rank-sum tests were applied to identify significant CNA (P < 0.01) and all results were adjusted for multiple hypothesis testing using the Benjamini–Hochberg procedure. Complete lists of CNAs and somatic small variants are available in Tables SI and SII respectively. Validation of CNA in chromosome 8p was performed in tumour and germline samples with the TaqMan RT-PCR assays for copy number: Hs00497516_cn (DOK2) and Hs00132843_cn (TNFRSF10B), and expression: Hs00929587_m1 (DOK2) and Hs00366278_m1 (TNFRSF10B) (Thermo Fisher Scientific, Waltham, MA, USA).

CNA analysis identified del8q in all five patients, including homozygous deletions in three of them (IBR2, IBR3 and IBR4) at relapse. In IBR2, a heterozygous del8q at baseline evolved to homozygous loss at the time of ibrutinib progression. IBR3 demonstrated a subclonal homozygous deletion in a third of the tumour population at baseline that increased at the time of disease progression (Fig 1A and Figure S1). While del8q is present in up to half of WM patients at diagnosis, it is usually heterozygous. The progressive expansion of tumour clones with homozygous del8q in patients developing ibrutinib resistance is of great interest since many important regulators of signalling impacted by ibrutinib reside within this domain. Among these are the inhibitor of BTK (IBTK), inhibitors of MYD88/NF-κB (TNFAIP3, HIVEP2, TRAF3IP2, IRAK1BP1) signalling, as well as important regulators of apoptosis (FOXO3, BCLAF1, PERP) (Guerrera et al., 2018). We also observed del8p in 4/5 (80%) patients at ibrutinib progression with the remaining patient (IBR1) having a microdeletion as well. Del8p was present at baseline in two patients (IBR1 and IBR2), and absent in the other (IBR3). Genes lost in del8p patients included BLK, DOK2, and TNFRSF10A/B. BLK plays an important role in B-cell proliferation and differentiation and is a target of ibrutinib (Berglöf et al., 2015). DOK2 is a negative regulator of TLR/MyD88 signalling, and loss of DOK2 triggers ERK1/2 signalling in lipopolysaccharide-stimulated cells (Shinohara et al., 2005), similar to the observed increased in ERK1/2 signalling in BTK<sup>Cys481→Arg</sup>-mutated WM and activated B-cell-like diffuse large B-cell lymphoma cells (Chen et al., 2018). In the patient with the del8p microdeletion (IBR1), TNFRSF10A/B, but not DOK2 or BLK, were impacted. While the size of the deletion was unchanged at progression, this patient did acquire a truncating mutation in DOK2. Del8p was also observed in 3/5 CLL patients who progressed on ibrutinib and underwent WES (Burger et al., 2016). Abrogation of apoptotic signalling related to loss of TRAIL receptor (i.e., TNFRSF10A and TNFRSF10B) was suspected to contribute to ibrutinib resistance. We therefore decided to validate our findings and analyse the expression of two of these genes (DOK2 and TNFRSF10B) in patients progressing versus patients responding to ibrutinib therapy (very good partial response, VGPR). Although the high frequency of del8p was confirmed in 3/5 patients for DOK2 and 4/5 for TNFRSF10B (Figure S2), no differences were observed regarding expression of these genes between patients progressing compared to patients with continued response (Figure S3). Additional studies into the functional significance of this event in a larger population are warranted. Larger studies may also clarify whether the presence of these alterations at baseline have an impact on the depth of response to ibrutinib, similarly to what was observed for MYD88 and CXCR4 mutations.

In two patients (IBR1 and IBR3), we also found chromosome 3q amplifications that encompassed a copy number neutral loss of heterozygosity region of approximately seven million base pairs that included TBL1XR1, a transcriptional regulator of NF-κB and WNT signalling (Jung et al., 2017). Mutations of TBL1XR1 are the most frequent variant found in MYD88<sub>Wt-type</sub> WM patients (Hunter et al., 2018), who show poor responses to ibrutinib monotherapy (Treon et al., 2019), though no somatic mutations in TBL1XR1 were identified in this study. No other recurrent CNAs were detected, though two patients (IBR4 and IBR5) displayed multiple deletions or gains involving large chromosomal regions (Fig 1A and Figure S1).

Samples taken at ibrutinib progression showed a high proportion (median 87%; range 81–89%) of acquired versus persistent mutations (median 13%, range 11–19%). Most persistent mutations (60%) maintained the same allele frequency from baseline and did not enrich with the acquired mutations, suggesting that a high level of clonal heterogeneity persisted from baseline despite long exposure to ibrutinib therapy. Acquired mutations were more subclonal than persistent alterations (median allele frequency of 8.4% vs. 13.9%, P = 0.08). Among variants acquired at progression,
Table I. Clinical and biological characteristics of Waldenström macroglobulinaemia patients progressing on ibrutinib.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>IBR1 Baseline</th>
<th>IBR1 Progression</th>
<th>IBR2 Baseline</th>
<th>IBR2 Progression</th>
<th>IBR3 Baseline</th>
<th>IBR3 Progression</th>
<th>IBR4 Baseline</th>
<th>IBR4 Progression</th>
<th>IBR5 Baseline</th>
<th>IBR5 Progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>77</td>
<td>–</td>
<td>61</td>
<td>–</td>
<td>66</td>
<td>–</td>
<td>78</td>
<td>–</td>
<td>73</td>
<td>–</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>–</td>
<td>Male</td>
<td>–</td>
<td>Male</td>
<td>–</td>
<td>Male</td>
<td>–</td>
<td>Male</td>
<td>–</td>
</tr>
<tr>
<td>Serum IgM (mg/dl)</td>
<td>4130</td>
<td>NA</td>
<td>3630</td>
<td>NA</td>
<td>2490</td>
<td>NA</td>
<td>1300</td>
<td>NA</td>
<td>5790</td>
<td>3970</td>
</tr>
<tr>
<td>Serum IgM m-spike (g/dl)</td>
<td>2-21</td>
<td>1-12</td>
<td>2-09</td>
<td>NA</td>
<td>1-41</td>
<td>NA</td>
<td>0-98</td>
<td>0-81</td>
<td>3-62</td>
<td>NA</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>10-3</td>
<td>10-5</td>
<td>12-3</td>
<td>10-7</td>
<td>9-1</td>
<td>7-8</td>
<td>8-9</td>
<td>8-4</td>
<td>10-8</td>
<td>7-7</td>
</tr>
<tr>
<td>Platelets (x10^9/l)</td>
<td>230</td>
<td>83</td>
<td>265</td>
<td>133</td>
<td>142</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Serum β2-microglobulin (mg/l)</td>
<td>5</td>
<td>30</td>
<td>70</td>
<td>90</td>
<td>40</td>
<td>5</td>
<td>60</td>
<td>30</td>
<td>80</td>
<td>–</td>
</tr>
<tr>
<td>Bone marrow involvement (%)</td>
<td>Fludarabine,</td>
<td>–</td>
<td>Rituximab,</td>
<td>–</td>
<td>Rituximab</td>
<td>–</td>
<td>Cladribine,</td>
<td>–</td>
<td>–</td>
<td>Rituximab, chlorambucil, bendamustine</td>
</tr>
<tr>
<td>Prior therapies</td>
<td>rituximab, CPR, bendamustine-R, bortezomib/dex, tositumomab</td>
<td>–</td>
<td>ofatumumab,</td>
<td>C, pentostatin</td>
<td>–</td>
<td>–</td>
<td>C, rituximab, IFNalpha, bendamustine, bortezomib/dex</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Time on ibrutinib (months)</td>
<td>9-6</td>
<td>37-1</td>
<td>36-4</td>
<td>–</td>
<td>9-1</td>
<td>–</td>
<td>23-0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Best response to ibrutinib</td>
<td>PR</td>
<td>–</td>
<td>PR</td>
<td>–</td>
<td>VGPR</td>
<td>–</td>
<td>VGPR</td>
<td>–</td>
<td>PR</td>
<td>–</td>
</tr>
<tr>
<td>Other events supporting progression</td>
<td>Splenic enlargement</td>
<td>New pleural effusion</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MYD88 Status (% allele frequency)</td>
<td>L265P (36%) (47%)</td>
<td>L265P (94%) (100%)</td>
<td>S243N (85%) (79%)</td>
<td>NA</td>
<td>L265P (29%) (83%)</td>
<td>NA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>CXCR4 Status (% allele frequency)</td>
<td>Wild-type</td>
<td>–</td>
<td>S338X (62%) (41%)</td>
<td>D185Y (7%) Wild-type</td>
<td>NA</td>
<td>Wild-type</td>
<td>NA</td>
<td>S338fs (37%)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NA, not available; C, cyclophosphamide; P, prednisone; R, rituximab; dex, dexamethasone; IFN, interferon; PR, partial response; VGPR, very good partial response; fs, frameshift mutation present at this amino acid site.
BTK mutations were identified in three of five patients (IBR2, IBR4, and IBR5). Two of them (IBR2, IBR5) had BTK Cys481Ser mutations at the ibrutinib-binding domain, while the other patient (IBR4) had a BTK Thr62Ala mutation. Both BTK Cys481-mutated patients also harboured alterations in genes related to B-cell receptor signalling, such as PLCG2 Tyr495His, LYN Ala2Stop and LYN Ala139Thr, and CD79B Asp33Tyr, and were CXCR4-mutated.

In patients without BTK mutations, we searched for other alterations that could contribute to ibrutinib resistance. For this, we focused on variants likely to be deleterious based on the CADD score, present only at progression, and affecting genes expressed in WM. We identified several candidates including ITCHAla646Ser (n = 2), an ubiquitin ligase whose substrates are CXCR4, LYN or SYK; RNF19BArg30Gly (n = 2), another ubiquitin ligase involved in STAT1-mediated transcriptional activity; FCRL3 Glu944Gln (n = 1), a protein that modulates innate immune signalling in B cells; BIRC2 Ala506Glu (n = 1), a regulator of alternative NF-κB, and MAPK signalling; and negative regulators of TLR signalling including TOLLIP, seen in two patients as TOLLIPArg228His and TOLLIPArg228His and DOK2Tyr345Stop in one patient (IBR1) (Fig 1B). In another patient (IBR3), a truncating SYK Tyr526Stop at baseline was not detectable at time of progression on ibrutinib. Since SYK Tyr526 is a critical phosphorylation site required for BCR activation and substrate recruitment, this clonal evolution could signify enrichment of clones capable of functional BCR/SYK signalling (Bohnenberger et al., 2011).

These studies provide novel insights into clonal evolution of ibrutinib-resistant WM. Ibrutinib-resistant WM is still an uncommon event. This limited the number of eligible study patients and precluded definitive analysis. While resistance in some samples can be explained by acquired alterations in BTK and PLCG2 (Xu et al., 2017), identification of alternative mechanisms of resistance for the rest of the population is crucial as the number of WM patients on ibrutinib for multiple years continues to grow. Key findings include the recognition that del6q and del8p may accompany ibrutinib resistance, a notable finding since these regions encompass many key regulators of BTK, MYD88/NF-κB, and apoptotic signalling. Moreover, these studies also identified recurring
mutations in ubiquitin ligases, innate immune signalling, and TLR/MYD88 pathway regulators in ibrutinib-resistant WM patients.

Acknowledgements

The authors would like to thank the Orszag Family Fund for WM Research, Peter S. Bing M.D., the International Waldenstrom’s Macroglobulinemia Foundation, and the Leukemia and Lymphoma Society.

Funding

ZRH was supported by a National Institutes of Health Development Award (Spore 5P50CA100707-12), and an American Society of Hematology Scholar Award. CJ is supported by a grant from the FEHH-Fundación CRIS.

Author contributions

CJ, SPT, and ZRH designed the study and wrote the manuscript. RGS and AW reviewed manuscript and provided critical input. CJ, GGC, and ZRH conducted the bioinformatic analysis. LX, NT, AK, MGD, JC, XL, MM, and GY performed tumour cell selection, DNA isolation and Sanger analysis. SPT and JJC provided patient care, obtained the samples, and collected the clinical data.

Conflicts of interest

ZRH, SPT, RGS, JJC have received consulting fees, and/or research funding from Pharmacyclics Inc., Janssen Inc., and/or AbbVie Inc.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. List of copy number alterations observed in five Waldenström macroglobulinemia patients progressing on ibrutinib. Significant CNA (P < 0.01) according to Kolmogorov-Smirnov and Wilcoxon rank-sum tests are displayed.

Table SII. Somatic small variants of Waldenström macroglobulinemia patients resistant to ibrutinib. List of variants present in at least one patient of the cohort. Only somatic coding variants and with a CADD score ≥20 are displayed.

Fig S1. B-allele frequency of the copy number abnormalities in Waldenström Macroglobulinemia patients progressing on ibrutinib. Predicted B-allele frequency profiles of the five patients at baseline and progression. Gains and losses are shown in red and blue, respectively.

Fig S2. Copy number validation of del8p. Copy number assays on TNFRSF10B (A) and DOK2 (B) were run in quadruplicate in tumor (at the time of progression) and germline samples of the 5 patients. Results confirmed the findings in all patients except for IBR4, whose copy number was normal for both genes. IBR1 showed deletion of TNFRSF10B but not of DOK2, in accordance with the whole-exome sequencing data. Additional validation was performed in 6 patients (V1-6) who came off ibrutinib but after the WES analysis was complete. Only one of the six (V3) was positive for TNFRSF10B and DOK2 deletion. After reviewing the medical records, this patient proved to be the only patient who progress while on ibrutinib while the others came off for intolerance or lack of response. He was wild-type for BTK Y481 mutation and had progressed after one year on ibrutinib.

Fig S3. Gene expression by RQ-RCR of TNFRSF10B (A) and DOK2 (B). Expression of both genes was assessed at baseline and progression in 2 of the patients progressing on ibrutinib (IBR1 and IBR3) and compared to 3 patients who are currently responding to this therapy (very good partial response, VGPR) and to healthy donors (B-cells and memory cells). Y-axis represents fold change, which was measured relative to a calibrator (*), i.e., the lowest expresser of the gene, whose expression by definition is 1. No differences were observed between patients progressing vs. non-progressing in either of the two genes.

References


