bjh short report

Genomic evolution of ibrutinib-resistant clones in Waldenström macroglobulinaemia

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Summary

Ibrutinib is highly active in Waldenström macroglobulinaemia (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 Macroglobulinaemia (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 $MYD88^{wild\text{-}type}$ 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 (BTK^{Cys481}), 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 $BTK^{wild\text{-}type}$ 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.

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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 del6q in all five patients, including homozygous deletions in three of them (IBR2, IBR3 and IBR4) at relapse. In IBR2, a heterozygous del6q 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 del6q is present in up to half of WM patients at diagnosis, it is usually heterozygous. The progressive expansion of tumour clones with homozygous del6q 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^{Cys481}-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 *TNFRSFB*) 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 *TNFRSFB* (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 variants found in $MYD88^{wild\text{-}type}$ 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.

	IBR1		IBR2		IBR3		IBR4		IBR5	
Characteristics	Baseline	Progression	Baseline	Progression	Baseline	Progression	Baseline	Progression	Baseline	Progression
Age (years)	77	–	61	–	66	–	78	–	73	–
Gender	Male	–	Male	–	Male	–	Male	–	Male	–
Serum IgM (mg/dl)	4130	NA	3630	NA	2490	NA	1300	NA	5790	3970
Serum IgM m-spike (g/dl)	2·21	1·12	2·09	NA	1·41	NA	0·98	0·81	3·62	NA
Haemoglobin (g/dl)	10·3	10·5	12·3	10·7	9·1	7·8	8·9	8·4	10·8	7·7
Platelets ($\times 10^9$/l)	230		83		265		133		126	
Serum β_2-Microglobulin (mg/l)	4·5		2·5		5·6		14·2		3·9	
Bone marrow involvement (%)	30	70	70	90	40		5	60	30	80
Prior therapies	Fludarabine, rituximab, CPR, bendamustine-R, bortezomib/dex, tositumomab	–	Rituximab, ofatumumab, C, pentostatin	–	Rituximab	–	Cladribine, C, rituximab, IFNalpha, bendamustine, bortezomib/dex	–	Rituximab, chlorambucil, bendamustine	–
Time on ibrutinib (months)	9·6	–	37·1	–	36·4	–	9·1	–	23·0	–
Best response to ibrutinib	PR	–	PR	–	VGPR	–	VGPR	–	PR	–
Other events supporting progression						Splenic enlargement New pleural effusion				
MYD88 Status (% allele frequency)	L265P (36%)	(47%)	L265P (94%)	(100%)	S243N (85%)	(79%)	NA	L265P (29%)	NA	L265P (83%)
CXCR4 Status (% allele frequency)	Wild-type	–	S338X (62%)	(41%)	D185Y (7%)	Wild-type	NA	Wild-type	NA	S338fs (37%)

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.

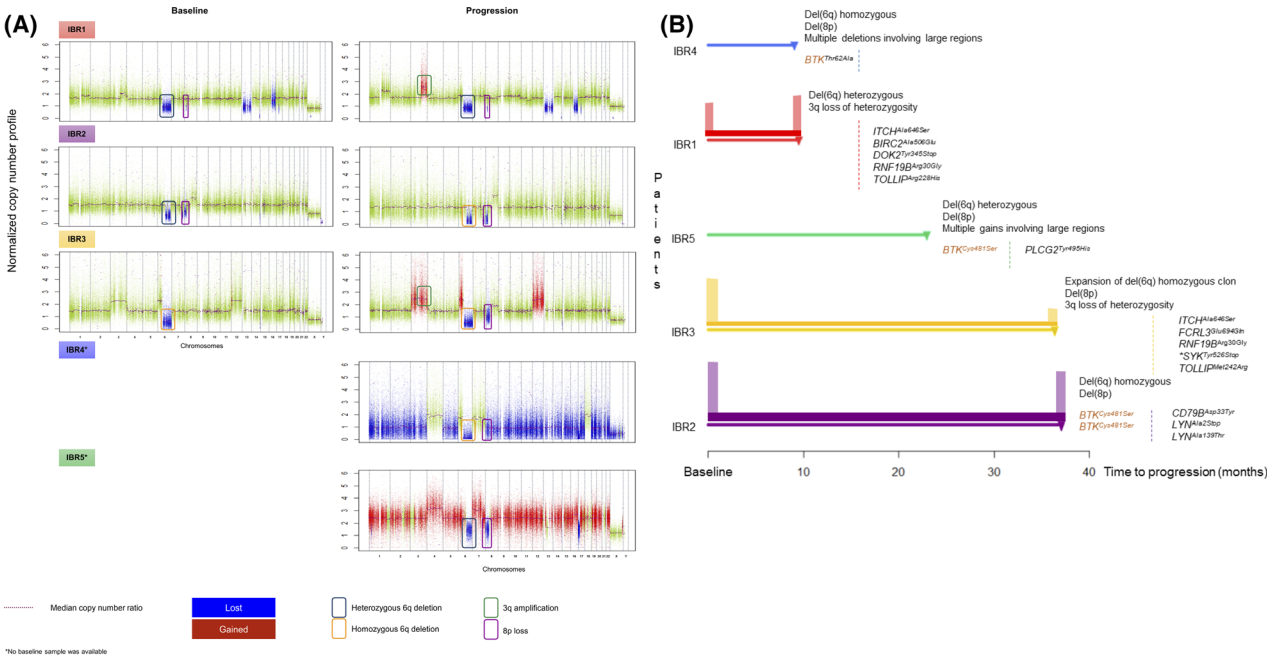

Fig 1. Copy number and genomic alterations identified by whole-exome sequencing in Waldenström Macroglobulinaemia (WM) patients progressing on ibrutinib. (A) Copy number alterations in WM patients progressing on ibrutinib. Each square represents a chromosome. Losses are coloured in blue, gains in red, and green corresponds to normal copy number. Deletion of chromosome 6q was present in 100% patients at baseline and progression, including homozygous loss in 33% cases at baseline and in 60% at the time of ibrutinib progression. In IBR3 the homozygous deletion was inferred to be subclonal, affecting up to a third of the tumour population at baseline and increasing at progression. Del8p was observed in 2/3 cases (66%) at baseline and in 4/5 cases (80%) at progression, and includes BLK, and DOK2. The patient without del8p (IBR1) harboured a truncating mutation in DOK2. Finally, 3q amplification was absent at baseline, but present in 40% patients at the time of progression. (B) Depicts genomic alterations in WM patients progressing on ibrutinib. Patients (represented with lines) are displayed by time to progression. For IBR1, IBR2 and IBR3, the proportion of persistent mutations (horizontal bars) is shown with respect to the mutations present at only one of the time points (vertical bars). On the right, the most remarkable alterations are shown for each patient: first, recurrent copy number variations and then, somatic mutations. Patients with mutated *BTK*^{Cys481} also harboured other mutations which affect the B-cell receptor pathway. Other recurrently mutated genes were also identified (i.e. *ITCH, RNF19B* and *TOLLIP*), as well as copy number alterations, such as 3q amplification with loss of heterozygosity, with an embedded uniparental disomy that included *TBL1XR1* in the two patients lacking *BTK* mutations.

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 *ITCH*^{Ala646Ser} (n = 2), an ubiquitin ligase whose substrates are CXCR4, LYN or SYK; *RNF19B*^{Arg30Gly} (n = 2), another ubiquitin ligase involved in STAT1-mediated transcriptional activity; *FCRL3*^{Glu694Gln} (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 *TOLLIP*^{Met242Arg} and *TOLLIP*^{Arg228His}, and *DOK2*^{Tyr345Stop} 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.

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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 sequencing. 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 SI. 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^{Cys481} 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.

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