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Date:
2019-09-01

Citation:
Blombery, P., Birkinshaw, R. W., Nguyen, T., Gong, J. N., Thompson, E. R., Xu, Z., Westerman, D. A., Czabotar, P. E., Dickinson, M., Huang, D. C. S., Seymour, J. F. & Roberts, A. W. (2019). Characterization of a novel venetoclax resistance mutation (BCL2 Phe104Ile) observed in follicular lymphoma. British Journal of Haematology, 186 (6), pp.e188-e191. https://doi.org/10.1111/bjh.16069.

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Article type : Letters

**Characterization of a novel venetoclax resistance mutation (BCL2 Phe104Ile) observed in follicular lymphoma**

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/BJH.16069

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Keywords: BCL2, lymphomas, genetic analysis

Venetoclax is an oral, highly selective BCL2 inhibitor with significant clinical efficacy in a range of B-cell lymphoproliferative disorders, including chronic lymphocytic leukaemia (CLL)(Roberts*, et al* 2016, Seymour*, et al* 2017), mantle cell lymphoma (MCL)(Davids*, et al* 2017), multiple myeloma (MM)( Moreau*, et al* 2017) and follicular lymphoma (FL)(Davids*, et al* 2017). Whilst various mechanisms of potential resistance to this targeted agent have been reported in model systems( Fresquet*, et al* 2014, Jayappa*, et al* 2017, Punnoose*, et al* 2016), to date only upregulation of BCL-xL (also termed BCL2L1) (Agarwal*, et al* 2018) or acquisition of a specific *BCL2* mutation (Gly101Val)(Blombery*, et al* 2019) have been observed in samples from patients with progressive MCL or CLL respectively during therapy and confirmed as causing clinical resistance. Venetoclax monotherapy induces objective responses in 38% of patients with FL, including 14% complete responses(Davids*, et al* 2017). To date, mechanisms of secondary resistance in FL patients treated with venetoclax remain undefined. We describe a patient with relapsed/refractory FL treated with venetoclax who, after an initial response, developed progressive disease on venetoclax therapy through the emergence of a subclone harbouring multiple acquired *BCL2* mutations, including a novel candidate resistance mutation which reduced venetoclax binding – BCL2 Phe104Ile.

A 55-year-old man was enrolled on a Phase 1 clinical trial of single agent venetoclax (NCT01328626; Roberts*, et al* 2016)) for treatment of symptomatic relapsed FL. At study entry he had bilateral fluorodeoxyglucose (FDG)-avid inguinal lymphadenopathy and right tonsillar involvement on $^{18}$FDG-positron emission tomography (PET) scan. After approximately four months on venetoclax (1200 mg/day) he achieved a complete metabolic response on PET scan. After 29 months on therapy he had progressive FDG-avid right inguinal lymphadenopathy which was biopsied, confirming recurrent FL without evidence of large cell transformation. He subsequently ceased venetoclax. Given the

presence of an activating *EZH2* mutation he was treated with an EZH2 inhibitor (tazemetostat) and is in ongoing complete response at 34 months on therapy.

Targeted next generation sequencing (Supplementary Methods) was performed on FL biopsy samples immediately prior to venetoclax treatment and at disease progression on venetoclax (Table SI). In addition to typical pathogenic mutations observed in FL (*CREBBP*, *KMT2D* and *EZH2*) there was evidence of aberrant somatic hypermutation (aSHM) of the *BCL2* gene with presence of multiple mutations in the 5'untranslated region and the first coding exon. Copy number variation (CNV) and structural variation (SV) analysis identified a focal copy number loss of *TP53* and an *IGH-BCL2* translocation present in both the pre- and post-venetoclax samples. No new CNVs or SVs were detected in the post-venetoclax sample.

In the post-venetoclax sample, seven *BCL2* mutations were detected that were not detectable in the pre-venetoclax biopsy (sensitivity 3-5% variant allele frequency [VAF]). Five of the newly acquired *BCL2* mutations were predicted to result in amino acid changes in the BCL2 protein and one mutation (c.585+13G>A) was predicted to result in a Gly200Ser in a non-canonical *BCL2* transcript (NM_000657.2).

Strikingly, one of the mutations acquired during venetoclax treatment was predicted to result in a phenylalanine substitution to an isoleucine at amino acid 104 of the BCL2 protein, which is located at the venetoclax binding site of BCL2. The VAF of the Phe104Ile mutation (12.3%) was the highest of the newly acquired *BCL2* mutations in the post-venetoclax sample (estimated cancer cell fraction approximately 50% based on immunohistology) consistent with its presence in a significant proportion of the tumour compartment. The mutation could not be detected by droplet digital polymerase chain reaction (sensitivity 0.1%) in the pre-venetoclax sample. Whilst the Phe104Ile has not been described before in cancer (COSMIC) or population databases (gnomAD), two other substitutions at this position (Phe104Leu and Phe104Cys) have been previously reported in a lymphoma cell line rendered resistant through continuous exposure to venetoclax. Functional evidence to date for these *BCL2* mutations indicates resistance to the drug due to reduced venetoclax binding(Fresquet*, et al* 2014).

Another acquired *BCL2* mutation in this patient (Gly33Arg) has been described in FL but it is unrelated to venetoclax therapy and experimental data to date suggests it has increased affinity for the pro-apoptotic proteins BIM (BCL2L11) and PUMA (BBC3) in protein binding studies and in cellular models(Correia*, et al* 2015).

Phase analysis of paired sequencing read data from the pre- and post-venetoclax samples were consistent with the emergence of a new clonal lineage, characterized by the presence of Gly33Arg and Phe104Ile mutations within the same allele, arising from a Leu23Val negative stem clone (Figure 1). Based on the previous observation that *BCL2* mutations are highly enriched in the *IGH* translocated allele(Correia*, et al* 2015), the newly acquired *BCL2* mutations were assumed to occur in the heterozygous state. The observed VAFs of the newly acquired *BCL2* mutations were consistent with ongoing aSHM in the Gly33Arg/Phe104Ile mutated clone giving rise to subclones containing addition mutations - Ala4Thr, Arg6Lys, Arg12Trp and c.-2G>A (Figure 1, Table SI).

Given the previous observations of acquired resistance to venetoclax and altered drug binding as a result of Phe104 mutations(Fresquet*, et al* 2014), we focussed on whether the clinically observed Phe104Ile mutation also confers resistance to venetoclax. RS4;11 cells overexpressing the mutant were over 40-fold less sensitive to venetoclax than RS4;11 BCL2 WT cells (Figure 2A, Supplementary methods). We then investigated the binding of the Phe104Ile mutant to both venetoclax and pro-apoptotic proteins in Surface plasmon resonance binding experiments (Figure 2B, Supplementary methods). While its affinities for BIM or BAX BH3 peptides were unaltered, we observed marked impairment of venetoclax binding to the Phe104Ile mutant to a similar magnitude as that observed with the Gly101Val and Phe104Leu (approximately 300-fold decrease in affinity, $K_i$ = 5.9 nM and 0.018 nM for Phe104Ile and WT respectively)(Blombery*, et al* 2019).

Whilst any of the newly observed *BCL2* mutations in our patient may have contributed to clinical resistance, our experimental data strongly suggest the Phe104Ile as an important candidate resistance mutation in this patient. Moreover, its binding to venetoclax or apoptotic regulators is similar to that observed with the adjacent Gly101Val resistance mutation previously described in CLL(Blombery*, et al* 2019).

In summary, we have described the emergence of a novel candidate *BCL2* mutation in a patient with FL treated with venetoclax which is associated with significantly reduced venetoclax binding to its target and is sufficient to confer cellular resistance. Our observations in this case provide a strong rationale for investigating the role of acquired *BCL2* mutations as a result of aSHM as a venetoclax resistance mechanism in patients with follicular lymphoma.

**Acknowledgements –** We gratefully acknowledge the funding support of the Snowdome Foundation, Wilson Centre for Lymphoma Genomics, Vision Super, the Leukaemia Lymphoma Foundation and the Australian National Health and Medical Council.

**Contributions –** PB designed research, analysed data and wrote the paper. RWB/TM/JNG/ZX performed experiments and analysed data. MD/JFS provided clinical care to the patient. ERT/PEC/DCSH/AWR analysed data and wrote the paper. DAW wrote the paper. All authors reviewed and approved the final manuscript.

**Disclosure of Conflicts of Interest -** RWB, JNG, ZX, PEC, DCSH and AWR are employees of the Walter and Eliza Hall Institute, which receives milestone and royalty payments related to venetoclax and distributes a fraction of these to employees based on previous venetoclax-related research. JFS has received research funding from AbbVie and Genentech and is a consultant and is a member of advisory boards for both companies. DCSH has received research funding from Genentech. AWR has received research funding from AbbVie.



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**Figure 1 – Inferred clonal hierarchy based on *BCL2* mutation profile in follicular lymphoma biopsies pre- and post-venetoclax therapy**

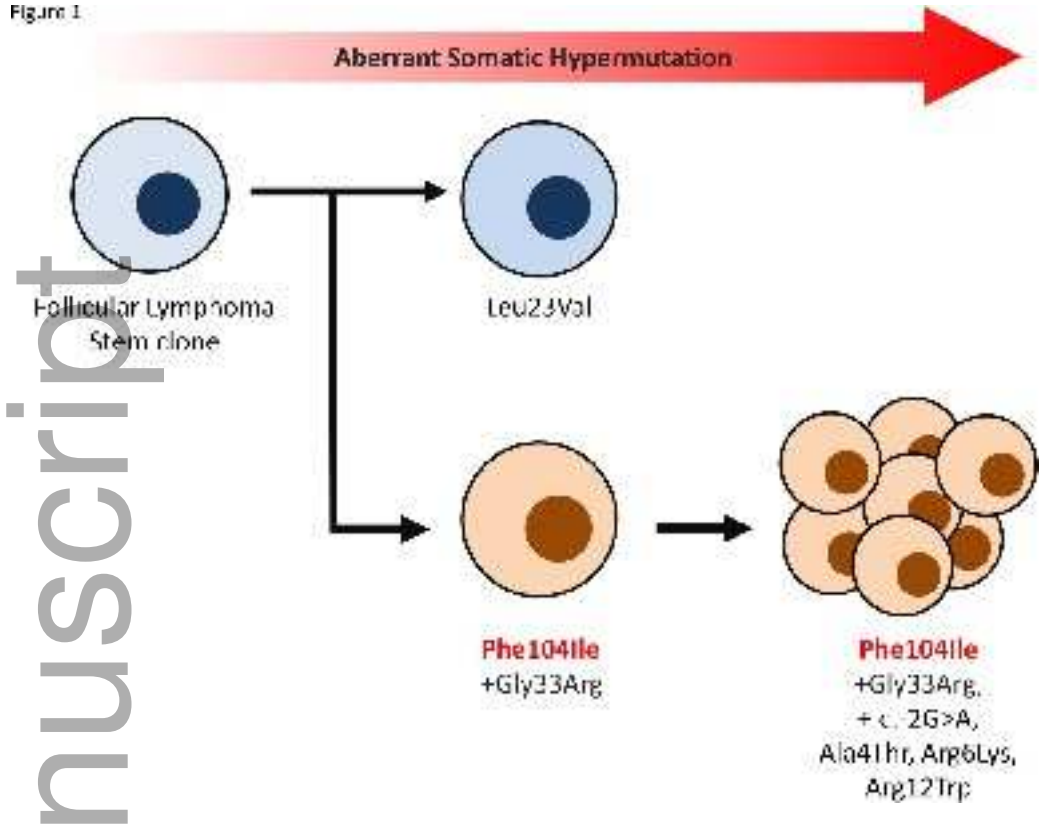
Clonal relatedness defined by observations on informative *BCL2* genomic variants where phasing could be determined by sequence read analysis (c.-17C>T, Glu13Asp, c.6G>A, Thr56Met, c.-83C>T, Leu23Val, Phe104Ile, Gly33Arg, c.-2G>A, Arg6Lys, Ala4Thr, Arg12Trp). In the pre-venetoclax sample, the clone bearing the Leu23Val mutation and its precursors including a stem clone were dominant, collectively comprising approximately 90% of tumour compartment (blue clones). The Phe104Ile-containing clone could not be detected in the pre-venetoclax biopsy. In the post-venetoclax sample, the representation of the Leu23Val-containing clone was reduced relative to the newly emergent Phe104Ile-containing clone and its derivatives (brown clones). Variants in different alleles were assumed to be present in different clones. *BCL2* NM_000633.2.

**Figure 2 - Attenuated sensitivity to venetoclax and reduced drug binding as a result of *BCL2* Phe104Ile mutation**

(A) Expression of BCL2 Phe104Ile in the RS4;11 cell line reduces sensitivity to venetoclax. The BCL2 Phe104Ile mutant or wildtype (WT) BCL2 were equally expressed (left panel), and the *in vitro* sensitivities to venetoclax (0-10 µM; right) measured 24 h later.

(B) BCL2 Phe104Ile protein binding affinities for BimBH3 peptide, BaxBH3 peptide or venetoclax (from left to right) as determined by surface plasmon resonance. $K_D$ BaxBH3 1.4 nM and 2.8 nM, $K_D$ BimBH3 0.15 nM and 0.29 nM, for BCL2 Phe104Ile and wild-type respectively. BimBH3 and BaxBH3 peptide affinities were determined by direct binding with raw data (coloured) and fits (black) shown. Venetoclax affinity was determined by competition surface plasmon resonance, with steady-state response plotted against BCL2 concentration (0-250 nM) at 3 different venetoclax concentrations (0-60 nM) with associated curve fits (black) shown. The $K_i$ for Phe104Ile of 5.9 nM was markedly reduced compared to that observed in control experiments with WT BCL2 ($K_i$ = 0.018 nM; data not shown). Graphs are representative images from one of two independent experiments and affinity constants mean values from the two independent experiments.

Figure 1

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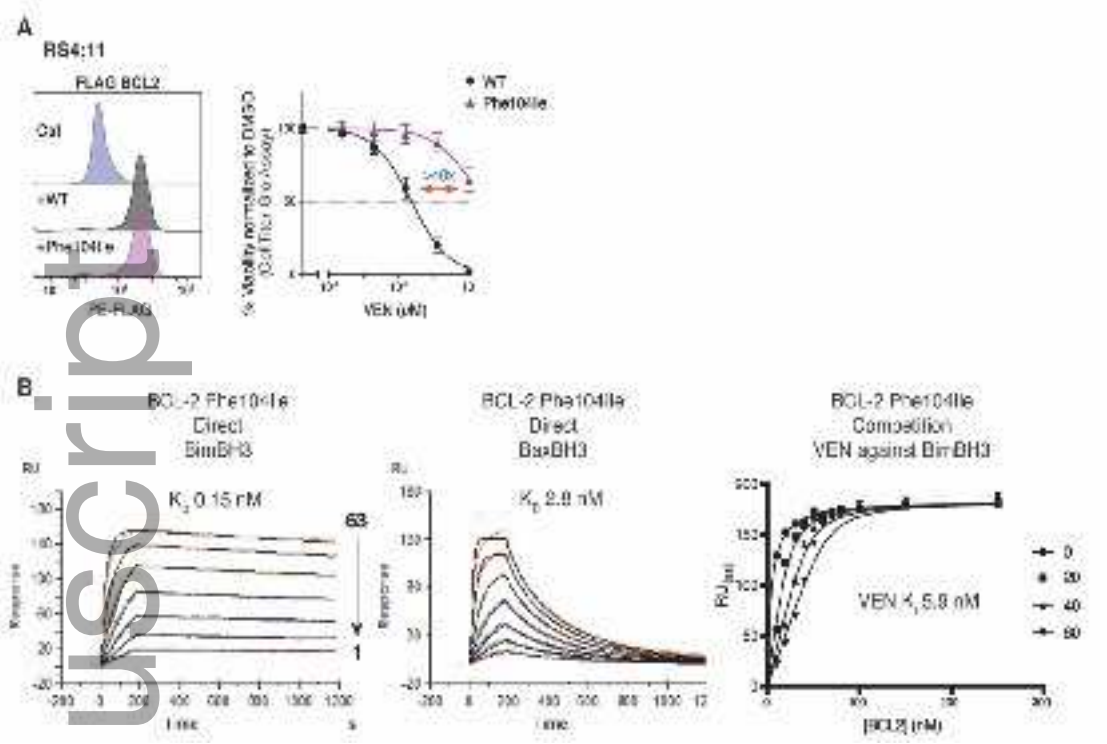


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