**Multiple BCL2 mutations co-occurring with Gly101Val emerge in chronic lymphocytic leukemia progression on venetoclax**

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**Supplementary Material**

*Cohort description*

The patient cohort of the first 67 consecutive patients with relapsed chronic lymphocytic leukemia/small lymphocytic lymphoma treated with venetoclax on three early phase clinical trials at our institutions has been previously described[1]. All provided written informed consent, and the studies were conducted in accordance with the Declaration of Helsinki and after Human Research Ethics Committee approval. Outcome data were updated to October 1, 2019. Twenty-eight patients in the cohort have demonstrated CLL progression at time of analysis (compared to 21 at previous description[2]). CLL-progression for this cohort was defined as formal progression meeting iwCLL criteria[3] or a progressive increase in measurable residual disease in peripheral blood or bone marrow sufficient to trigger intervention by the treating clinician (either addition of rituximab to venetoclax or allogeneic stem cell transplantation). 26/28 patients had suitable samples for *BCL2* Gly101Val ddPCR mutation testing. 11/26 (42%) patients had detectable *BCL2* Gly101Val mutations by droplet digital PCR. 7/11 have been described previously (CLL2, CLL3, CLL5, CLL6, CLL8, CLL12, CLL14)[2]. The additional four patients with Gly101Val mutations consisted of three patients that had CLL progression since the previous description (CLL16, CLL17, CLL20)[2] and one patient that had progressive CLL at the time of the previous description and tested negative in a sample at disease progression with low disease

burden (CLL11). Gly101Val was subsequently detected in this patient on samples after further disease progression with higher tumor burden.

**Supplementary Methods**

*Droplet digital PCR – assay design*

Droplet digital PCR targeting Gly101Val was performed as previously described[2].

A multiplex droplet digital (ddPCR) assay targeting *BCL2* Asp103Glu (NM_000633.2:c.309C>A) and Asp103Tyr (NM_000633.2:c.307G>T) was designed using forward primer 5'-CCGGTGCCACCTGTG-3', reverse primer 5'-GCTGGCTGGACATCTCG-3' and allele specific probes containing locked nucleic acid (LNA) bases to enhance specificity against wild-type (WT) and mutant sequence. WT and mutant probes for Asp103Glu were 5'-AGA+A+G+TC+G+TC+GC-3' and 5'-AGA+A+T+TC+GT+CG+CC-3' respectively. WT and mutant probes for Asp103Tyr were 5'-AG+T+C+GTCG+CC-3' and 5'-AAG+T+A+GT+CG+CC-3' respectively. These probes contained either HEX or 6-FAM as reporter dye and a 3' Iowa Black® FQ as quencher.

A ddPCR assay targeting *BCL2* Val156Asp (NM_000633.2:c.467T>A) was designed using forward primer 5'-TACCCAGCCTCCGTTATCC-3', reverse primer 5'-TGGAGGAGCTCTTCAGGG-3' and allele specific probes containing LNA bases against WT and mutant sequence. WT and mutant probes for Val156Asp were 5'-ACACAT+G+A+CCCCAC-3' and 5'-ACACAT+G+T+CCCCAC-3 respectively. WT probe contained HEX and mutant probe contained 6-FAM as reporter dye, both contained 3' Iowa Black® FQ quencher.

A ddPCR assay targeting *BCL2* Arg107_Arg110dup (NM_000633.2:c.319_330dup) was designed using forward primer 5'-CTCAAAGAAGGCCACAATCC-3', reverse primer 5'- CATCTTCTCCTCCCAGCC-3' and a hydrolysis probe specific for the mutant allele 5'-TAGCGGCGGCGGTAG-3' that was tagged with reporter dye FAM and double quencher ZEN™/Iowa Black™ FQ. DNA input was controlled by measuring the number of copies of *RPP30* gene using the Bio-Rad RPP30 assay.

*Droplet digital PCR – procedure*

Each 25 µL ddPCR reaction of *BCL2* Asp103 assay contained 12.5 µL of ddPCR™ SuperMix for probe (No dUTP) (Bio-Rad), 2 µL of 360 GC enhancer (Applied Biosystems), 900 nM of forward and reverse primers, 500 nM of *BCL2* Asp103Glu WT and mutant probes, 250 nM of *BCL2* Asp103Tyr WT and mutant probes and 50 ng template DNA.

Each 25 µL ddPCR reaction of *BCL2* Val156Asp and *BCL2* Arg107_Arg110dup contained 12.5 µL of ddPCR™ SuperMix for probe (No dUTP) (Bio-Rad), 2 µL of 360 GC enhancer (Applied Biosystems), 900 nM of forward and reverse primers, 250 nM of WT and mutant probes and 50 ng template DNA.

Reactions were prepared in a semi-skirted twin.tec® 96 well plate (Eppendorf). Following droplet generation on the QX-200 Droplet Generator (Bio-Rad), the plate was sealed with a pierce-able foil heat seal (Bio-Rad) and PCR performed on a C1000 Touch™ thermal cycler (Bio-Rad). PCR condition was: 10 minutes at 95°C, 40 cycles of 30 seconds at 94°C and 1 minute at 58°C for Asp103 assay or 57.5 °C for Val156Asp/ Arg107_Arg110dup assay, 10 minutes at 98°C and hold at 4°C. Each step had a ramp rate of 2°C/second. Droplets were measured on the QX200 Droplet Reader (Bio-Rad) and data was analyzed with QuantaSoft software v1.0.596 (Bio-Rad). The threshold for discrimination between positive droplets from negative droplets was set manually with an adequate distance from the background as ascertained by no template and WT controls.

*Digital next generation sequencing*

Genomic DNA was extracted from peripheral blood and/or bone marrow aspirate for next generation sequencing (NGS)-based testing. Unique molecular index (UMI) based amplicon libraries were prepared using the standard protocol for QIAseq targeted DNA panel (QIAGEN) as per manufacturer's specifications. Pooled libraries were sequenced on an Illumina NextSeq 500 on a NextSeq 500/550 Mid-Output v2.5 kit (300 cycles; Illumina) using a 151-cycles paired-end protocol. Alignment and

variant calling was performed using the QIAGEN CLC Genomic Workbench (v12.0.2) using the QIAseq DNA Somatic Variant (Illumina) workflow. All variant calls were manually inspected in integrated genome viewer (IGV) ver2.6.3.

*Hybridization-based next generation sequencing analysis*

Indexed libraries were sequenced on an Illumina NextSeq (paired-end 75 bp reads). After base calling and de-multiplexing, a Seqliner-framework analysis pipeline was used to align reads to the human reference genome (GRCh37 assembly) using BWA-MEM, followed by marking of duplicate reads, base quality score recalibration, local indel realignment and variant calling using GATK Haplotype Caller (https://software.broadinstitute.org/gatk/). In addition, aligned sequence data was processed through a dedicated bioinformatics pipeline which included variant calling with GATK4/Mutect2 (https://software.broadinstitute.org/gatk/) in order to improve detection of low level acquired variants. In addition alignments were processed to obtain a count of the number of occurrences of each base at each position in the coding region of *BCL2*. Per-position base counts were combined to produce a baseline per-position distribution across a cohort of approximately 1000 samples. Individual sample per-position counts were processed to compute the Kullback–Leibler (KL) divergence between the sample and the global counts for every coding nucleotide. The KL-divergences for each position were collated and used to estimate the parameters for the gamma distribution which were used to compute a p-value for the hypothesis that the distribution of counts at that position is the same as the global distribution at that position. Copy number analysis was performed using on and off target reads from this hybridization-based NGS panel as described previously[4,5].

*Surface Plasmon Resonance Binding experiments*

Experiments were performed as previously described[6]. Briefly, BCL2 protein mutants were engineered into a soluble BCL2 construct with a modified α1-2 loop as previously described[7]. using a BIAcore S200 using a SA sensor chip (GE Healthcare) immobilized with biotinylated BIMBH3 peptide, with BIMBH3-

4A peptide as a nonbinding reference. Peptide affinities were determined by direct binding with BCL2 (0–40 nmol/L) as the analyte. Venetoclax affinity was determined by competition against immobilized BIMBH3 peptide, using BCL2 (0–250 nmol/L) premixed with four concentrations of venetoclax (0-60 nmol/L) as the analyte. Direct binding experiments were fitted to a 1:1 binding site model for BIMBH3 binding or to a steady-state competition model for compound binding as previously described[6].

*Functional assays of the BCL2 mutants in human cell lines*

The FLAG-tagged Gly101Val or Asp103Glu BCL2 constructs were made and introduced into human B-lineage cell line RS4;11 and KMS-12-PE as previously described[2]. To test sensitivity to venetoclax or navitoclax, the cells were seeded in 96-well plate at 5,000 cells/well and treated with serial diluted concentrations (0-10 μM, 5-point 1:8 dilution) of the drugs. Cell viability 24 h after treatment was then determined using the CellTiter-Glo assay (Promega, Cat#G9241).

**Supplementary Table 1 – *BCL2* mutations detected by NGS in patients harboring *BCL2* Gly101Val mutations**

| Patient ID | Mutation Detected | Cancer Cell Fraction (%)* | TP53 abnormality** | Prior lines of therapy |
|---|---|---|---|---|
| CLL2 | *BCL2* c.302G>T; p.(Gly101Val) | 16.8 | Yes | 5 |
| | *BCL2* c.307G>T; p.(Asp103Tyr) | 1.6 | | |
| | *BCL2* c.319_330dup; p.(Arg107_Arg110dup) | 5.9 | | |
| | *BCL2* c.386G>T; p.(Arg129Leu) | 1.7 | | |
| | *BCL2* c.467T>A; p.(Val156Asp) | 1.6 | | |
| CLL3 | *BCL2* c.302G>T; p.(Gly101Val) | 68.4 | Yes | 3 |
| | *BCL2* c.319_330dup; p.(Arg107_Arg110dup) | 4.9 | | |
| | *BCL2* c.467T>A; p.(Val156Asp) | 2.4 | | |
| CLL5 | *BCL2* c.302G>T; p.(Gly101Val) | 36.4 | No | 3 |
| | *BCL2* c.308A>T; p.(Asp103Val) | 5.3 | | |
| | *BCL2* c.309C>A; p.(Asp103Glu) | 1.6 | | |
| | *BCL2* c.338C>G; p.(Ala113Gly) | 13.5 | | |
| | *BCL2* c.386G>T; p.(Arg129Leu) | 22.2 | | |
| | *BCL2* c.467T>A; p.(Val156Asp) | 3.9 | | |
| CLL6 | *BCL2* c.302G>T; p.(Gly101Val) | 2.3 | Yes | 3 |
| | *BCL2* c.302_303delinsTT; p.(Gly101Val) | 0.74 | | |
| | *BCL2* c.307G>T; p.(Asp103Tyr) | 3.2 | | |
| | *BCL2* c.308A>T; p.(Asp103Val) | 1.4 | | |
| | *BCL2* c.309C>A; p.(Asp103Glu) | 0.6 | | |
| | *BCL2* c.338C>G; p.(Ala113Gly) | 0.53 | | |
| | *BCL2* c.467T>A; p.(Val156Asp) | 0.8 | | |
| CLL8 | *BCL2* c.302G>T; p.(Gly101Val) | 1.9 | Yes | 8 |
| | *BCL2* c.307G>T; p.(Asp103Tyr) | 9.0 | | |
| | *BCL2* c.309C>A; p.(Asp103Glu) | 4.6 | | |
| | *BCL2* c.319_330dup; p.(Arg107_Arg110dup) | 2.1 | | |
| | *BCL2* c.467T>A; p.(Val156Asp) | 9.6 | | |
| CLL11 | *BCL2* c.302G>T; p.(Gly101Val) | 0.1 | Yes | 7 |
| | *BCL2* c.319_330dup; p.(Arg107_Arg110dup) | 0.6 | | |
| CLL12 | *BCL2* c.302G>T; p.(Gly101Val) | 9.2 | Yes | 5 |
| | *BCL2* c.307G>T; p.(Asp103Tyr) | 30.6 | | |
| | *BCL2* c.309C>A; p.(Asp103Glu) | 10.2 | | |
| CLL14 | *BCL2* c.302G>T; p.(Gly101Val) | 4.3 | No | 4 |
| CLL16 | *BCL2* c.302G>T; p.(Gly101Val) | 7.3 | No | 3 |
| | *BCL2* c.319_330dup; p.(Arg107_Arg110dup) | 0.5 | | |
| | *BCL2* c.338C>G; p.(Ala113Gly) | 29.1 | | |
| CLL17 | *BCL2* c.302G>T; p.(Gly101Val) | 56.2 | No | 1 |
| | *BCL2* c.307G>T; p.(Asp103Tyr) | 20.2 | | |
| CLL20 | *BCL2* c.302G>T; p.(Gly101Val) | 0.08 | No | 2 |
| | *BCL2* c.467T>A; p.(Val156Asp) | 4.3 | | |

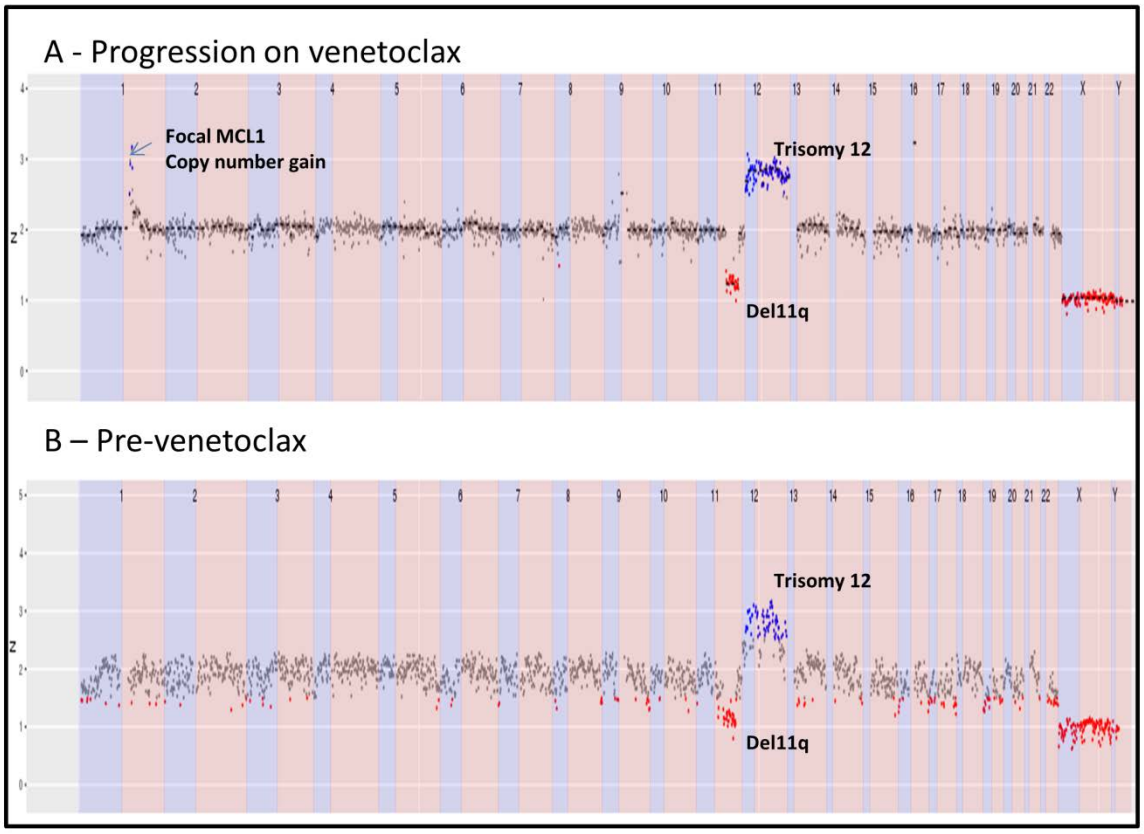
***Cancer cell fraction (CCF) calculated as variant allele frequency divided by disease burden determined by flow cytometry x 2 (assuming heterozygosity). *BCL2* NM_000633.2 **TP53 abnormality defined as pathogenic mutation or del17p detectable by FISH prior to venetoclax therapy**

## Supplementary Table 2 – Kinetic parameters for SPR experiments

| BCL2 protein | BIMBH3 on rate ($M^{-1}$ $s^{-1}$ ± SD, *n=3*) | BIMBH3 off rate (s-1 ± SD, *n=3*) | BIMBH3 $K_D$ (nM ± SD, *n=3*) | Ven $K_I$ (nM ± SD, *n=3*) |
|---|---|---|---|---|
| WT † | 4.1 x$10^5$ ± 0.8 | 1.1 x$10^{-4}$ ± 0.5 | **0.29** ± 0.17 | **0.018** ± 0.014 |
| Gly101Val † | 4.8 x$10^5$ ± 2.4 | 4.1 x$10^{-4}$ ± 2.3 | **0.84** ± 0.04 | **3.2** ± 1.1 |
| Asp103Glu | 1.0 x$10^6$ ± 0.2 | 1.3 x$10^{-4}$ ± 0.6 | **0.12** ± 0.03 | **0.33** ± 0.05 |

Data are from three independent experiments +/- 1 standard deviation. †previously reported[6]

**Supplementary Figure 1**




(A) Copy number analysis from patient CLL16 demonstrating focal *MCL1* copy number gain on chromosome 1q as well as trisomy 12 and del 11q present in sample at progression on venetoclax (B) *MCL1* amplification not detectable in pre-venetoclax sample from patient CLL16.

**References**

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