Supplementary Information

Cell Cycle Reprogramming for PI3K Inhibition Overrides Relapse-Specific C481S BTK Mutation Revealed by Longitudinal Functional Genomics in Mantle Cell Lymphoma

Running title: Overriding relapse-specific BTK mutation in MCL

Key words: Ibrutinib, PD 0332991, AKT

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Supplemental information

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Supplementary Methods

Immunohistochemistry.

Immunohistochemistry was performed as previously described (2). Briefly, 4-μm sections of paraffin-embedded bone marrow, lymph node or spleen tissues were processed using a TechMate500 BioTek automated immunostainer and reagents (Ventana Medical Systems) according to manufacturer's specifications. Pax 5 was detected using an anti-Pax 5 mAb (Serotec) and a red chromogen. The nuclei were visualized by counterstaining with hematoxylin. Simultaneous expression of other proteins was detected with monoclonal antibody to Ki67 (Zymed), cyclin D1 or Rb and polyclonal rabbit antibody to phospho-Ser807/811 or phospho-Ser780 of human Rb (Cell Signaling).

As a control for immunoglobulin synthesis, serial sections were stained with rabbit antibody for IgM, IgD, IgG, IgA, Igk, or Igl (DakoCytomation). The frequency of MCL (Pax 5^{+}) cells expressing a specific protein was scored by image analysis, using the Leica Biosystems, Ariol SL50 system. $p < 0.05$ were considered statistically significant.

Immunofluorescence staining of p65 subcellular localization

JEKO-1 cells were treated with or without PD 0332991, ibrutinib and anti-IgM for the time indicated, then cytospun on a glass slide, fixed with 3.7 % formaldehyde in PBS, and

permeabilized with 0.05 % saponin in PBS containing 10% FBS. After incubation for 1 hour with anti-human p65 antibody (1:50; sc-109, Santa Cruz Biotechnology), cells were washed with PBS containing 10 % FBS and incubated for 30 min with a PE-conjugated anti-rabbit antibody (1:300; Jackson ImmunoResearch Laboratories). Cells were counterstained with DAPI (Sigma). Images at a magnification of × 40 were captured using a Zeiss Axiovert 200 WideField fluorescent inverted microscope and analyzed with MetaMorph software.

Immunoblotting.

Proteins were analyzed with the following antibodies: CDK4 (#12790), cyclinD1 (#2926), AKT (#9272), AKT-pS473 (#9271), BTK-pY223 (#5082), PLCγ2-pY759 (#3874), PLCg2 (#3872), Cyclin B (#4135), CDK1 (#9112), c-Myc (#9402_N-term) and p110α (#4249) from Cell Signaling Technology; PI3Kδ (p110δ, sc-7176), actin (sc-1615) Cyclin A (sc-751), p27 (sc-528), p52 (sc-298) and NIK (sc-7211) all from Santa Cruz Biotechnology; BTK (ab32555) and V5-tag (ab9116) from Abcam.

shRNA interference.

Cells were infected with lentiviruses containing shRNA specific for *PIK3IP1* (#1: TRCN0000135363; #2: TRCN0000135429), or *LacZ* (TRCN0000072229) (The RNAi Consortium at Broad Institute, Cambridge, MA). Knockdown was confirmed by quantitative RT-PCR at 60-72 h post-transduction or by immunoblotting.

Lentiviral infection for stable expression of human BTK^{WT} and BTK^{C481S}

pLX304-BTK^{WT} (V5 tagged) was obtained from DNASU plasmid repository (http://dnasu.org/DNASU/), BTK^{C481} construct (6xHis tagged) was described previously (3) and pLX304-GFP (V5 tagged) from the RNAi Consortium at Broad Institute, Cambridge, MA. Stably infected DT40 cells were selected with Blasticidin S (10 μg/mL, Life Technology) 24 hours after lentiviral infection. BTK protein expression was confirmed by immunoblotting using BTK and V5 antibodies and the C481S mutation was confirmed by Sanger sequencing.

Sanger sequencing

Genomic DNAs were isolated from MCL primary sample using the DNeasy blood and tissue kit (Qiagen). Primers for PCR amplification of samples were obtained from Life Technology:

BTK: 5'-AGTTGTATGGCGTCTGCACCAA-3' (forward)

5'-AGGTCTCGGTGAAGGAACTGCT-3' (reverse).

PLCG2: 5'-CCAAGGCTTTCAGAAACCCC-3' (forward)

5'-ACAGGGTGTAGTCATTGGGG-3' (reverse).

Sanger sequencing was performed at the Institute of Biotechnology of Cornell University.

Targeted sequencing

Tissue from a pleural effusion biopsy of Pt2 at ibrutinib relapse was submitted as a formalin fixed, paraffin embedded (FFPE) block to a CLIA-certified, CAP-accredited laboratory (Foundation Medicine, Cambridge, MA). Hybridization capture of 3769 exons from 236 cancer related genes and 47 introns of 19 genes frequently rearranged in cancer was applied to >50 ng DNA extracted from the block and sequenced to a median coverage of 486x. The most significant mutations are detailed in the Supplementary Table S4.

Computational Analysis of the WES and WTS data

For the computational analysis of the somatic SNPs both present in WES and WTS datasets, we developed our own scripts in Ruby and Awk. We used ONCOTATOR (http://www.broadinstitute.org/oncotator/) for their corresponding annotation and we further characterized the relevant mutations using SIFT, POLYPHEN2 and PROVEAN. For the Copy Number Variations analysis, we correspondingly developed our own Python scripts.

Quality check of WES data. We used the freely available software FASTQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) to make a quality check of our WES data. While the per base Quality scores of the 75bps reads were above 30 on average, we also observed the there were big non random fluctuations in the per base content of the reads in the

first 15 bases of the 5' end (due to the use of primers in the exome capture technique). For this reason, we decided to create an alternative and rather conservative WES data set, for comparison, were we performed trimming of 15 bases at the 5' end and 2 bases at the 3' end. For the trimming, we used the freely available software FASTX –Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/).

Quality check of WTS data. We also used the freely available software FASTQC to make a quality check of our WTS data. While the per base Quality scores of the 50bps reads were above 30 on average, we also observed the there were big non random fluctuations in the per base content of the reads in the first 12 bases of the 5' end (due to the use of primers in the RNA-seq process). We then also decided to create an alternative and rather conservative WTS data set, for comparison, were we performed trimming of 12 bases at the 5' end and 2 bases at the 3' end. The trimming was done simultaneously with the alignment using STAR (4).

WES data alignment. The corresponding raw *fastq* files from DNA-seq (from both untrimmed and trimmed sets) were aligned to the GRCh37 hg19 human genome. BWA (5) was used, which in our experience outperforms other common aligners for WES data.

WES data variant calling. The subsequent variant calling of the aligned data was done with the latest GATK version 2.5 (6) with base quality score recalibration, indel realignment, duplicate removal, and performing SNP and INDEL discovery and genotyping across all the 5 samples simultaneously, using standard hard filtering parameters and variant quality score recalibration (7).

WTS data alignment. The raw *fastq* files from RNA-seq were aligned also to the GRCh37 hg19 human genome. We aligned the data using two different aligners: BWA , for the untrimmed data, and STAR (4), for the trimmed data. The first one was performed within the pipeline of the commercially available software GeneSifter (Geospiza) and the second one as part of our own pipeline.

WTS data variant calling. The variants of the un-trimmed aligned data (BWA) were called, within the pipeline of GeneSifter, with GATK version 2.2. For the trimmed aligned data (STAR) we used Samtools to call the variants. The reason why in the latter case we did not use GATK was that the per base map quality scores given by STAR are in a different scale as required from GATK. While these scores could be fixed to fulfill GATK's requirements, we would have introduced bias and therefore loss precision in the posterior variant calling process.

Analysis and filtering. From the recalibrated variant calling file (vcf), we kept only those variants that fulfilled the following criteria:

- The variants in the tumor samples should not be in the control samples (PBCs).
- The variants in the tumor samples should have Read Depth different than 0 in the Alternate Allele allowing, thus, for very low frequencies.
- Further impose Phred Score of the called variant (Q) bigger or equal than 20 and passing all the filters; total read depth of the called variant (RD) bigger or equal than 10 and genotype quality of the called variant (GQ) bigger or equal than 20.

Gene expression analysis by WTS To determine mRNA abundance, we used the bioinformatic program Genesifter (Geospiza) which used BWA and mapped read normalization. Values are represented as RPKM (Reads per kilo base per million) and were normalized to the expression of *Actin-beta* (ActB)

Copy Number Variation analysis of Pt 1

XHMM (eXome-Hidden Markov Model) version 1.0 was used with default parameters, as per the software's manual, for all samples simultaneously (8). This involved using GATK (6) to calculate depth of coverage and GC content, and PLINK/SEQ to get the fraction of repeat-masked bases. From GATK's depth of coverage output, we calculated the average autosomal coverage of all samples, shown below:

SAMPLE	**average autosomal coverage**

CS	29.84
p_Ib1	72.35
p_Ib2	128.83
p_Ib3	49.97
r_IbBM	65.48
r_IbSP	100.48

LogR ratios of read depth were calculated as follows for the normal sample:

and **(B)** genes frequently mutated in the BCR signaling pathway and an in other lymphomas (11).

Supplementary Figure Legends

Figure S1. MCL cell lines are differentially sensitive to killing and cell cycle control by ibrutinib

A, cells from 4 MCL cell lines were cultured with ibrutinib at indicated concentrations for 72 hours. Cell death was determined by ToPro-3 staining and FACS analysis. **B,** cell cycle analysis (BrdU/PI double staining) of MCL cells cultured with ibrutinib for 72 hours (1 μmol/L). BrdU labeling was done for 30 minutes before analysis. Number in the FACS profile indicates the percentage of cells in sub-2N and S phase. Data are representative of 3 independent experiments.

Figure S2. NIK and p52 of the alternative NF-κB pathway are expressed in JEKO-1 MCL cells and not modulated by targeting of CDK4 with PD 0332991 in combination with ibrutinib

A, immunoblotting of NIK and p52 in JEKO-1 cells cultured in the presence or absence of PD 0332991 (0.3 μmol/L; 24 hours) before addition of ibrutinib (1 μmol/L; 24 hours) and goat anti

human IgM (anti-IgM, 5 μg/mL). **B,** immunoblotting of NIK and p52 in JEKO-1 cells cultured with PD 0332991 (0.3 μmol/L) for time indicated.

Figure S3. pG1 reprogramming for PI3K inhibition overrides relapse-specific C481S BTK mutation

A, immunoblotting of DT40 BTK-/- cells before and after infection with with a lentivirus expressing human BTK^{WT}-V5 or BTK^{C481S}-6xHis. **B**, schema for incubation of DT40_ BTK^{WT} or DT40_BTK^{C481S} cells in the absence or presence of PD 0332991 (0.3 μmol/L) for 16 hours before addition of GS-1101, GDC-0941 or BAY 80-6946 for 32 hours in the continuous presence of PD 0332991. **C,** DT40_ BTK^{WT} and DT40_BTK^{C481S} cells were cultured in the absence (Cntl) or presence of PD 0332991 and PI3K inhibitors as shown in **B**. Cell death was determined by ToPro-3 staining and FACS analysis. The total number of live cells was determined by Trypan Blue staining (x 20,000 cells per mL). Error bars represent SD. Data are representative of 3 independent experiments.

Supplemental References

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