Biological and clinical implications of *BIRC3* mutations in chronic lymphocytic leukemia

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SUPPLEMENTARY APPENDIX

BIOLOGICAL AND CLINICAL IMPLICATIONS OF *BIRC3* MUTATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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SUPPLEMENTARY METHODS

Cell studies
The human CLL cell line MEC1, the SMZL cell lines SSK41, VL51, and the MCL cell lines MAVER-1, Z-138 and JEKO-1 were cultured under standard conditions in RPMI-1640 with L-glutamine supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin (Sigma Aldrich). Human HEK-293T cells were maintained in Iscove's Modified Dulbecco Medium (IMDM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin and 2mM L-glutamine (Sigma Aldrich) under identical conditions.
Three primary cells samples known to harbor heterozygous inactivating mutations of *BIRC3* were included in the experiments. Two *BIRC3* wild type cases were used as controls.

Western blot analysis
The entire non-canonical NF-κB pathway was assessed using the following specific primary antibodies: anti-BIRC3 (Cell Signaling, #3130), anti-TRAF2 (Cell Signaling, #4712), anti-TRAF3 (Cell Signaling, #4729), anti-MAP3K14 (Cell Signaling, #4994), anti-Phospho-NF-κB2 p100 (Cell Signaling, #4810), anti-NF-κB2 p100/p52 (Cell Signaling, #4882). Anti-β-actin (Sigma Aldrich, #A2066) was used as loading control. The Qproteome Nuclear Protein Kit (Qiagen) was used according to the manufacturer's instructions to isolate nuclear proteins from cells. Anti-β-tubulin (Sigma Aldrich, #T5201) and anti-BRG1 (G-7) (Santa Cruz Biotechnology, #17796) were used as controls for the purity of the cytoplasmic and nuclear fractions, respectively. Horseradish peroxidase-conjugated goat anti-mouse (LI-COR, #926-80010) or anti-rabbit (LI-COR, #926-80011) antibodies were used to highlight binding by enhanced chemiluminescence with the Clarity Western ECL Substrate (Biorad). Image acquisition and densitometric analyses were performed using the Molecular Imager Gel Doc XR System and the Quantity One software (Biorad).

RNA extraction and gene expression profiling
Total RNA was extracted from exponentially growing cell lines by TRIzol reagent (Life Technologies), and retro-transcribed using the Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was conducted with the Step One Plus apparatus (Step One software 2.0; Applied Biosystems) using commercially available TaqMan Gene expression assays (TNFAIP3: Hs00234713_m1; NFKB2: Hs00174517_m1; NFKBIA: Hs00153283_m1; NFKBIE: Hs00234431_m1; PLEK: Hs00950975_m1; WNT10: Hs00228741_m1; IL2RG: Hs00953624_m1; RELB: Hs00232389_m1; MALT1: Hs01120052_m1) (BIRC3: Hs00985031_g1) (Life Technologies). Reactions were done in triplicate from the same cDNA (technical replicates). The comparative CT method (ΔΔCT) was used to calculate relative expression levels of the gene under analysis, using GAPDH (Hs03929097_g1) as internal references.

Knockdown of MAP3K14 by RNA interference
Lentiviruses expressing 3 short hairpin RNAs (shRNAs) targeting MAP3K14, as well as the scrambled shRNA, were produced and cloned into the BamHI/HindIII cloning sites of the pGFP-C-shLenti vectors (OriGene Technologies). Within the 5'-LTR and 3'-LTR regions, each pGFP-C-shLenti vector contains an shRNA expression cassette driven by an U6 promoter, a puromycin resistance marker driven by a SV40 promoter and a GFP driven by a CMV promoter. The shRNA expression cassette consists of 29 bp target-gene-specific sequence, a 7 bp loop, and another 29 bp reverse complementary sequence, followed by a TTTTTT termination sequence. The HEK293T cell line was co-transfected with expression (3 different pGFP-C-MAP3K14-shLenti or pGFP-C-non-effective-shLenti) vectors and adjuvant vectors (pMDL, REV and VSV-G). Fluorescence microscope was utilized to check the expression of the GFP in the transfected HEK293T cell line. After virus titration, the VL51 cell line was infected with lentiviruses harboring the shRNAs against MAP3K14 and the scrambled through a spinoculation protocol. After four days, infected cells were monitored by flow cytometry for the expression of the GFP and were selected by puromycin (1.5 μg/mL).

Inhibitor studies
Cells were put under starvation in RPMI 0.1% FBS 24h before treatment. Then they were seeded at 8000 cells per well in a 96-well U-bottom plate and treated with 1 μM, 5 μM and 10 μM of Ibrutinib (PCI-32765, Selleckchem) or vehicle (DMSO). Relative growth was determined by a Cell-Titer Glo (CTG) Luminescent Cell Viability Assay (Promega) 72h and 96h after treatment, according to the manufacturer's instructions and luminescence was quantified using a Victor X (PerkinElmer) multilabel reader. Treatments were done in triplicate (biological replicates).

***In vitro* drug responses in primary CLL cells**
Leukemic cells were purified using Ficoll-Hypaque (Sigma Aldrich) from peripheral blood (PB) of CLL patients. Staining with CD19 and CD5 confirmed that in all samples leukemic cells were >90%. Patients were then divided into *BIRC3* mutated (MUT) or wild-type (WT). *TP53* mutated samples (and *BIRC3* WT) were selected as positive control (i.e., cells intrinsically resistant to therapies). Cells were cultured in RPMI 10% FCS (200 μl, all reagents from Sigma) at a density of 5x106/ml and both dose- and time-dependent responses were analyzed. Specifically, CLL cells were exposed to fludarabine) for 24-48 hours. Fludarabine was used at 1-5-10-25 μM and venetoclax at 5-10-50-100-500-2000 nM.

Apoptosis assay
Drug-induced apoptosis was measured using the eBioscience™ Annexin V Apoptosis Detection Kit APC (ThermoFisher) following the manufacturer's instruction. Data were acquired using a FACSCanto II cytofluorimeter (BD Biosciences) and processed with DIVA v6.1.3 and FlowJo Version 9.01 (TreeStar).

FCR treated patients
The study was designed as a retrospective observational analysis from a multicenter cohort of 287 (275 with complete clinical and molecular data) untreated CLL receiving first-line therapy with FCR in 17 different hematological centers. The following biological material was collected: *i)* 280 tumor genomic DNA (gDNA) and 7 tumor RNA isolated from peripheral blood (PB) before treatment start; and *ii)* paired germline gDNA from saliva from 14 cases. Normal gDNA from 22 healthy donors was also used to set the experimental background of the deep next generation sequencing (NGS) approach. Tumor and normal gDNA was extracted according to standard procedures[1].Tumor RNA was extracted according to the TRIzol Reagent protocol (Ambion Life Technologies). The clinical database was updated in April 2018. Patients provided informed consent in accordance with local Institutional Review Board requirements and the Declaration of Helsinki. The study was approved by the Ethical Committee of the Ospedale Maggiore della Carità di Novara associated with the Amedeo Avogadro University of Eastern Piedmont (study number CE 67/14).

Cancer personalized profiling by deep sequencing (CAPP-seq)
A targeted resequencing gene panel [2] was designed to include: *i)* coding exons plus splice site of 24 CLL genes known to be implicated in CLL pathogenesis and/or prognosis; *ii)* 3'UTR of *NOTCH1*; and *iii)* enhancer and promoter region of *PAX5* [3,4] (size of the target region: 66627bp). Tumor and germline gDNA were quantified using the Quant-iT™ PicoGreen dsDNA Assay kit (ThermoFisher Scientific) and 400 ng were sheared through sonication (Covaris M220 focused-ultrasonicator) before library construction to obtain 200-bp fragments. The size of the DNA fragments was checked using the Bioanalyzer (Agilent Technologies). The NGS libraries for gDNA were constructed using the KAPA Library Preparation Kit (Kapa Biosystems) and NGS libraries for RNA were constructed using RNA Hyper Kit (Roche) following the manufacturer's instructions. Hybrid selection was performed with the custom SeqCap EZ Choice Library (Roche NimbleGen). Multiplexed libraries (n = 10 per run) were sequenced using 300-bp paired-end runs on a MiSeq sequencer (Illumina).

Bioinformatic pipeline for variant calling after CAPP-seq
Initially, FASTQ sequencing reads were deduped. We deduped FASTQ sequencing reads from gDNA by utilizing the FastUniq v1.1 software, that collapses as duplicate reads only those fragments (read pairs) with 100% sequence identity that also share genomic coordinates. The same approach was also used to dedupe germline gDNA and normal gDNA from 22 healthy donors, to avoid the introduction of biases in variant calling due to the application of different deduplication protocols. Then, the deduped FASTQ sequencing reads were locally aligned to the hg19 version of the human genome assembly using the BWA v.0.6.2 software with the default setting, and sorted, indexed and assembled into a mpileup file using SAMtools v.1. The aligned read families were processed with mpileup using the parameters -A -d 10000000. For cases provided with paired germline gDNA, single nucleotide variations and indels were called in tumor gDNA vs germline gDNA, respectively, with the somatic function of VarScan2 using the parameters min-coverage 1 --min-coverage-normal 1 --min-coverage-tumor 1 --min-var-freq 0--min-freq-for-hom 0.75 --somatic-p-value 0.05 --min-avg-qual 20 --strand-filter 1 --validation 1. For cases lacking paired germline gDNA, single nucleotide variations and indels were called in tumor gDNA using the CNS function of VarScan2 using the parameters --min-coverage 0 --min-readge 2 --min-avg-qual 20 --min-var-freq 0 --min-freq-for-hom 0.75 --p-value 0.05 --strand-filter 1 --output-vcf 1 --variants 0. The variants called by VarScan 2 were annotated using the SeattleSeq Annotation 138 tool by using the default setting. Variants annotated as SNPs according to dbSNP 138 (with the exception of *TP53* variants that were manually curated and scored as SNPs according to the IARC TP53 database), intronic variants mapping > 2 bp before the start or after the end of coding exons, and synonymous variants were then filtered out. The following strict post-processing filters were then applied to the remaining variants to further improve variant call confidence. To filter out variants below the base-pair resolution background frequencies in gDNA across the selector, for cases provided with paired germline gDNA, the Fisher's exact test was used to test whether the frequency of the variant called by VarScan 2 was significantly higher from that called in the corresponding paired germline gDNA, after adjusting for multiple comparisons by Bonferroni test [multiple comparisons corrected p threshold = 0.00000018761163, corresponding to alpha of 0.05/(66627 x 4 alleles per position]. Accordingly, variants represented in > 10 reads of the paired germline and/or variants with a somatic p value from VarScan2 > 0.00000018761163 were no further considered. To filter out systemic sequencing errors, a database containing all germline and normal gDNA background allele frequencies was assembled. Based on the assumption that all background allele fractions follow a normal distribution, for both cases provided with paired germline gDNA and cases lacking paired gDNA, a Z-test was employed to test whether a given variant in the tumor gDNA differed significantly in its frequency from typical germline or normal gDNA background at the same position in all the other germline and normal gDNA samples, after adjusting for multiple comparisons by Bonferroni test [multiple comparisons corrected p threshold = 0.00000018761163, corresponding to alpha of 0.05/(66627 x 4 alleles per position]. Variants that

did not pass this filter were no further considered. Variant allele frequencies for the resulting candidate mutations and the background error rate were visualized using IGV.

Statistical analysis

Progression free survival (PFS) was the primary endpoint and was measured from date of treatment start to date of progression according to IWCLL-NCI guidelines (event), death (event) or last follow-up (censoring). Overall survival (OS) was measured from date of initial presentation to date of death from any cause (event) or last follow-up (censoring). Survival analysis was performed by Kaplan-Meier method and compared between strata using the Log-rank test. A false discovery rate approach was used to account for multiple testing, and adjusted p-values were calculated using the Bonferroni correction. A maximally selected rank statistic was used to determine the optimal cut-off for variant allele frequency (VAF) based on the Log-rank statistics. A cut-off of 3% of VAF was set for *TP53* mutations and of 10% for all the other genes. The adjusted association between exposure variables and PFS was estimated by Cox regression. Internal validation of the multivariate analysis was performed using a bootstrap approach to estimate means and confidence intervals of hazard ratios, and percentage of selection for each variable in the model. The number of bootstrap samples used was 1000. Apoptosis assays were analyzed using the two-way ANOVA test. All statistical tests were two-sided. Statistical significance was defined as p value < 0.05. The analysis was performed with the Statistical Package for the Social Sciences (SPSS) software v.24.0 (Chicago, IL), with R statistical package 3.1.2 and with GraphPad version 7 (GraphPad Software Inc).

SUPPLEMENTARY TABLES

Table S1. Target region

Table S2. Target region with ≥1000X and ≥2000X coverage

Table S3. 11q deletion, 17p deletion and *TP53* mutational status of tumor cell lines and primary CLL cells

Table S4. Primary cell lines divided into *BIRC3/TP53* mutated or wild-type (WT).

Table S5. Somatic non-synonymous mutations discovered in tumor samples

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