Zhang et al. Genetic characterization and drug sensitivity study of newly derived HGBL double/triple-hit lymphoma cell lines

Supplemental Information

Clinical summary of patients from whom the cell lines were derived

COH-DHL1 was derived from the tumor of a 58-year-old male with highly aggressive disease including circulating lymphoma cells. He was treated with R-EPOCH (R-CHOP + Etoposide phosphate) and alternating high dose cytarabine and high dose methotrexate, and then was treated with RICE (Rituximab + Ifosfamide + Carboplatin + Etoposide) for refractory disease and went into remission. The patient then underwent autologous stem cell transplantation but relapsed in 2 months and succumbed to the disease. At relapse, the tumor had a DLBCL morphology, and flow cytometry showed a monotypic B-cell population expressing CD10 dim, CD19, CD20, CD38, HLA-DR, and kappa light chain. Conventional cytogenetics demonstrated a complex karyotype showing 47,XY,ins(2;q)(q23;?),add(5)(p13),add(6)(q13),+7,del(9)(p22),t(8;14;18)(q24.1;q32;q21.1)[4]/46,XY[16] and FISH studies were positive for MYC/IGH fusion (66%) and BCL2 rearrangement (61.5%) and negative for BCL6 or CCND1 rearrangements.

COH-THL1 was derived from a 78-year-old male with a recent history of fever, night sweats, and circulating “blast-like” cells, thrombocytopenia and elevated LDH. A PET scan showed a small rib lesion and increased FDG uptake throughout the bone marrow (BM) consistent with involvement by malignancy. A BM biopsy showed involvement by a high-grade B-cell lymphoma, with triple-hit (MYC/BCL2/BCL6 translocations determined by FISH with BCL2/IGH and MYC/IGH fusion and BCL6 break-apart probes). Cytogenetics assay showed the following karyotype: 49,XY,+i(X)(p10),+Y, der(3)t(3;8;14) (q27.3;q24.21;32.33), der(8)t(8;14;3) (q24.21;q32.33;q27.3), t(8;14)(q24.21;q32.33), t(14;18)(q32.33;q21.3),+17[15]. The tumor cells were positive for CD19, CD20, CD10, BCL2, BCL6, and MYC (90%) and negative for TdT and CD34 by immunohistochemistry (IHC). Flow cytometry on the peripheral blood and BM confirmed the IHC findings and showed that the tumor cells were also positive for CD22, CD24, CD38, CD45, CD123 (dim), and surface kappa restricted which became lambda restricted at relapse after 5 months. A sample of blood was obtained for culture at relapse. The patient was
treated with R-EPOCH and venetoclax for 4 cycles. Approximately 6 months after diagnosis, the patient had relapsed disease. He was then treated with CD19-directed CAR T-cell therapy and has been in remission.

**Cell lines and culture conditions**

The DOGKIT cell line was purchased from Leibniz Institute / DSMZ-German collection of microorganisms and Cell cultures GmbH. COH-DHL1 and COH-THL1 were derived from patient samples at City of Hope Medical Center with informed consent. CS-THL1 was obtained from Dr. Serhan Alkan (Cedar Sinai Medical Center) with HGBL-TH reported previously. SU-DHL6 is a GCB-DLBCL cell line purchased from the American Type Culture Collection (Manassas, VA, USA). Jijoye cell line is a BL cell line with VNTR matching published data. The latter two are used for comparison with the four D/THL cell lines. All cell lines were maintained in RPMI 1640 medium (Gibco, LifeTechnologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (GE Healthcare Bio-Sciences, Pittsburgh, USA), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 I.U. penicillin, and 100 μg/ml streptomycin (Gibco) in a humidified 37°C incubator with 5% CO2.

For COH-DHL1 derivation, a cell suspension was prepared from the biopsy specimen of the lymphoma at the last relapse. The cells were cultured in RPMI-1640, supplemented with 20% FBS without any added cytokine. The cells were seeded at 10^5/well in a 24-well plate and kept at that concentration by splitting to additional wells when needed. When the cells were growing vigorously, they were transferred to 25-cm^2 flasks and split to keep the concentration below 10^6 per ml. The COH-DHL1 cells was later adapted to growth in RPMI-1640 medium supplemented with 10% FCS. Living cells were periodically frozen in 10% DMSO containing freezing medium and stored in the vapor phase of liquid nitrogen.

COH-THL1 cells were isolated from the peripheral blood using Ficoll-Hypaque density gradient centrifugation. The cells were washed and cultured similarly to COH-DHL1 with 10ng/ml of IL-4 added. After some time, these cells were adapted to grow without any cytokines in RPMI with 10% FBS. Cells were also frozen periodically. Cell growth rates and doubling times were determined by direct cell counting over 8 days in log phase.

**Cell line validation and characterization**

DNA isolated from the two COH cell lines were fingerprinted with short tandem repeat sequences using the GenePrint 10 System (Promega, Madison, WI) and compared to profiles in Cellosaurus.
Reference Database to confirm their uniqueness. Data from Applied Biosystems 3730 DNA Analyzer was analyzed with Genemapper (Thermo Fisher Scientific, Waltham, MA). To compare the cell lines with their 1-month and 2-month cell cultures as well as COH-THL1 original tumor, we performed flow cytometry with markers including CD45, CD20, CD19 or CD10, and kappa and lambda light chain (surface). We saw very similar phenotypes between the diagnostic case and the cell line (positive for CD45, CD19, and CD10).

**Subcutaneous inoculation to NSG mice**

Ten million cells of each COH cell line in 100 µl RPMI without serum and antibiotics were injected subcutaneously to a male NSG mouse at the age around 5 months. Three replicates were done for each of the two cell lines. The mice were sacrificed after three weeks, and tumors were collected and measured. Flow cytometry was performed for CD45, CD19 and immunoglobulin light chains. All animal procedures were in accordance with the guidelines and approved by the Administrative Panel on Laboratory Animal Care at City of Hope Comprehensive Cancer Center.

**Drugs and in vitro toxicity assay of the cell lines**

Drugs used in this study include the BCL2 inhibitor ABT-199, Bromodomain and extra terminal domain (BET) inhibitor JQ-1, CDK inhibitor Dinaciclib, dual PI3K and mTOR inhibitor BEZ235, BTK inhibitor PCI-32765, 20S proteasome inhibitor Bortezomib, MYC inhibitor 10074-G5, the RNA helicase eIF4A1 inhibitors Silvestrol, the CAMKIδ inhibitor Burbamine (BBM) and its derivative PA4. The newly developed drug BBM could bind and inhibit Ca2+/calmodulin-dependent protein kinase γ (CAMKIIγ) by targeting its ATP binding pocket. PA4 has much higher capacity to target the ATP binding pocket of both CAMKIIγ and CAMKIIδ. We tested them because CAMKIIγ was shown to stabilize c-Myc by phosphorylating at serine 62 (S62) and total c-Myc protein levels were reduced as a result of inhibition of CAMKIIγ and CAMKIIδ. JQ-1, Dinaciclib, BEZ235, PCI-32765 and Bortezomib were purchased from MedChem Express (Princeton, NJ). Silvestrol was kindly provided by Dr. Hans-Guido Wendel (Memorial Sloan Kettering Cancer Center, NY). Burbamine (BBM) and PA4 were prepared by Dr. Wendong Huang (City of Hope National Medical Center, CA).

Cells were plated in 96-well plates with 1 x 10^5 cells in 100 µl per well and exposed to serial drug dilutions for 72h. Cell viability was determined by CellTiter 96® AQueous One Solution Cell Proliferation Assay as described by the manufacturer (Promega Corporation, Madison, WI, USA). The absorbance was determined at 490 nm wavelength (OD490) by automated plate reader (Tecan Trading AG, Switzerland). Cell viability was expressed as a percentage of the untreated
viable cells. The average cell viabilities of four replicates at each concentration were fitted to sigmoidal dose–response curves with GRcalculator\textsuperscript{12}.

**High throughput sequencing**

Genomic DNA was extracted from the cell lines using DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD.). Libraries were prepared from genomic DNA. 250ng of genomic DNA was fragmented using Covaris S220 with the 200 bp peak setting. The fragmented DNA was end-repaired and ligated to Illumina adaptor oligo nucleotides with KAPA HyperPrep Kit (KAPA Biosystems, Wilmington, MA). Ligation products were purified and amplified with 7 cycles of PCR. The PCR products of COH-DHL1, COH-THL1, DOGKIT and CS-THL1 were captured for whole exome sequencing. Total RNA was isolated from COH-DHL1, COH-THL1, DOGKIT and CS-THL1 cell lines using RNeasy kit (Qiagen) following the manufacturer's protocol. RNA sequencing libraries were prepared with KAPA Stranded mRNA sequence Kit (Kapa Biosystems) according to the manufacturer's protocol. 100ng of total RNA from each sample were used for polyadenylated RNA enrichment with oligo dT magnetic beads, and the poly (A) RNA was fragmented with divalent cations under elevated temperature. First-strand cDNA synthesis was performed by reverse transcription and after second-strand cDNA synthesis, the double-stranded cDNA was end repaired, 3’ end adenylated, and ligated to bar-coded adaptors (Illumina, San Diego, CA). 12 cycles of PCR were performed to produce the final sequencing library. The libraries for COH-DHL1, DOGKIT and CS-THL1 are 100-nucleotide paired-end reads. COH-THL1 was sequenced as 50-bp single end reads. Both DNA and RNA sequencing were performed on Illumina HiSeq2500 platform according to the manufacturer’s guidelines.

**Gene expression analysis**

The fastq files were aligned with reference genome (hg38) with STAR. Raw read counts for each gene were then obtained after the bam files were sorted by coordinates. Gene expression was then analyzed in R environment by first normalizing with variance stabilizing transformation function in DEseq2(v1.14.1)\textsuperscript{13}, and then removing batch effect with ComBat function in SVA (v3.36.0)\textsuperscript{14}. We kept only the protein-coding genes and filtered the genes with normalized counts lower than 5, and genes in sex chromosomes and mitochondrial chromosome were further removed. A t-distributed stochastic neighbor embedding plot was made for normalized counts of the remaining genes. Top 5000 variable genes with highest median absolute deviation were selected and genes with Pearson correlation larger than 0.75 with the other genes were then removed to make a heatmap with ward D2 cluster method and correlation distance measure. GEP analysis of the four D/THL cell lines in our study was performed and for comparison, we also
analyzed RNAseq data of another 74 cell lines belonging to 11 different classes of lymphoma or leukemia from 18 different studies. The fastq files for these cell lines were collected from Sequence Read Archive (https://trace.ncbi.nlm.nih.gov/Traces/sra/) and Gene Expression Ominibus (https://www.ncbi.nlm.nih.gov/geo/). Only the samples without any treatment except DMSO were used in the analysis and their information are in Supplementary Table 1. Differential expression analyses were performed with DEseq2 normalized data using Gene Specific Analysis in Partek Flow (v10.0). The normalized data were then input into Gene Set Enrichment Analysis (GSEA)\textsuperscript{15} for pathway analysis and enrichment analysis in previously reported 104 D/THL signature genes\textsuperscript{16}.

**Mutation analysis**

Raw sequences were mapped to the reference genome (hg38) using Burrows-Wheeler Aligner (v0.7.17)\textsuperscript{18}. The bam files were sorted by coordinates. MarkDuplicates, RealignerTargetCreator, IndelRealigner, BaseRecalibrator, ApplyBQSR, and LeftAlignIndels in GATK (v4.1.9.0)\textsuperscript{19} were used for duplicate marking, local realignment, and base quality recalibration. Variants were called with Mutect2 in GATK for COH-DHL1 and THIT1 with matched normal control and tumor-only mode for the other samples. FilterMutectCalls was then applied to filter artifacts, which is followed by annotation with Funcotator (v1.7) in GATK. Silent mutations, and mutations with depth lower than 10, or variant allele depth lower than 4, or variant frequency lower than 0.05, were first removed. The remaining mutations were further filtered against germline mutations in 1000 Genomes Project database, dbSNP database (v153), and genome aggregation database (gnomAD). The mutations were analyzed and plotted with maftools\textsuperscript{20}. Mutations for four other GCB-DLBCL cell lines (DOHH2, VAL, SU-DHL4, and SU-DHL6) from public data (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-7722/) were called in the same way, and only those overlapping mutations with DepMap (https://depmap.org/portal/) database were kept. These four cell lines were reported to have double/triple hits\textsuperscript{21}, so we compared their genomic alterations with those of the four D/THL cell lines in our study.

**Analysis of copy number alterations**

Copy number alterations (CNA) was analyzed using an R package Copywriter [13] with a bin size of 100 kb based on non-targeted sequences, and further segmental analyses were performed within R statistical computing environment. We required that each CNA segment must contain at least 20 bins and have a segmental mean larger than log\textsubscript{2}(2.5/2) or 0.322 for CN gains, or less than log\textsubscript{2}(1.5/2) or -0.415 for CN losses. For cell lines that were derived from males, CNA of sex chromosomes with segmental mean larger than -0.415 or less than -2 were considered as CN
gains or CN losses, respectively. Recurrent gCNA were identified with GISTIC 2.0.23 at 95% confidence level and filtered with gold standard CNV from Database of Genomic Variants archive (https://www.ebi.ac.uk/dgva/). Genes in significant minimal common region (MCR) (FDR<0.05) were plotted together with mutations with maftools. These genes were filtered with a gene list expressed in normal B cells in which genes with FPKM larger than 2 were used for GO term analysis with enrichR (v3.0)²².

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Supplemental Figure 1. Fingerprinting results of the two COH cell lines with short tandem repeats. (A) Fingerprinting of COH-DHL1. (B) Fingerprinting of COH-THL1 line and its original tumor.

| Locus Name | Allele(s) for Tested Sample | Locus Name | Allele(s) for Tested Sample | Allele(s) for Tested Sample |
|------------|-----------------------------|------------|-----------------------------|-----------------------------|
|            | COH-DHL1                    | COH-THL1   | COH-THL1 Original           |
| AM         | X,Y                         | AM         | X,Y                         |
| CSF1PO     | 12,13                       | CSF1PO     | 10,11                       |
| D5S818     | 9,12                        | D5S818     | 12,13                       |
| D7S820     | 11,12                       | D7S820     | 10,11                       |
| D13S317    | 11,11                       | D13S317    | 13,13                       |
| D16S539    | 11,12                       | D16S539    | 13,13                       |
| D21S11     | 30,31.2                     | D21S11     | 28,32.2                     |
| TH01       | 9.3,9.3                     | TH01       | 6,9                         |
| TPOX       | 8,8                         | TPOX       | 8,8                         |
| vWA        | 14,18                       | vWA        | 16,17                       |
Supplemental Figure 2. Flow cytometry result of the COH-DHL1 (A) and COH-THL1 (B) cell line in Figure 1D when cultured for 1 and 2 months with CD45, CD20 and immunoglobulin light chains. Also included is flow cytometry of tumor at relapse for COH-THL1.
Supplemental Figure 3. Nonsynonymous mutations of expressed genes in the four D/THL cell lines. (A) Summary of the mutations showing number and distribution of mutations of different subtypes, as well as number of mutations for each cell line and each of the top 10 genes. Total mutation numbers or percentage of mutant samples are labeled on top of each bar in the bar plots. (B) Waterfall plot showing alteration of recurrent mutant genes in the four D/THL cell lines with different colors representing different types of mutations or CNA. The top bar plot shows the total number of mutations in each sample. The right bar plot shows number of different alterations for each gene labeled by the percentage of samples that have genetic alteration in the gene. “Multi_Hit” means genes with co-occurring mutations of different types. “Complex_Event” means genes with both mutations and CNAs.
Supplemental Figure 4. Gene expression profiling of D/THL cell lines and enrichment of 104 D/THL signature genes reported by Ennishi et al\textsuperscript{16}. (A) Gene expression heatmap of top variable 4364 genes among 78 cell lines including D/THL and other 11 types of leukemia/lymphoma cell lines with 46 previously reported DHL signature genes labeled. (B) Positive enrichment of D/THL vs. DLBCL+ BL in 48 DHL signature genes overexpressed in HGBL-D/TH patients. (C) Negative enrichment of D/THL vs. DLBCL+ BL in 56 DHL signature genes underexpressed in HGBL-D/TH patients.