Integrating genomic alterations in diffuse large B-cell lymphoma identifies new relevant pathways and potential therapeutic targets

Supplemental Material

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**Supplemental Methods**

**Gene Selection**

We selected 106 genes from previous whole exome sequencing studies of diffuse large B-cell lymphomas (DLBCL) and a large number of studies that analyzed the mutations of individual or small sets of genes in these lymphomas (Supplemental Table S2). In total, 3951 different genes were initially reviewed mainly from whole exome sequencing studies. Genes were selected according to the following criteria: Recurrent mutated genes in more than 10% of DLBCL in any of the studies, mutated
genes in which a somatic mutation was confirmed in at least 2 cases or annotated in COSMIC as somatic, and genes located on frequently altered regions in DLBCL. Three additional criteria were: functional evidence of the pathogenic role of the mutated gene in DLBCL, genes involved in pathways relevant for DLBCL, and drug target genes (45 genes were selected because they had been considered as drug targets in clinical trials according to the public database (http://www.cancer.gov/clinicaltrials). Some additional genes were added because they were reported in other lymphomas or could complement some of the pathways of interest (BLM, BRCA2, CCNH, FBXW7, HIST1H2BD, IDH1, ID3, MEF2C, MKI67, MPL, POU2F2, TCF3, SEMA5A, and WHSC1). We excluded several genes initially selected according to previous criteria for different reasons such as difficulties for primer design (e.g. P2RY8) or because they were known to be late replication genes, or because of their large size (PCLO, LRP1B, MUC16 and UNC5D) and the fact that they have been reported to accumulate large numbers of passenger mutations.33

Target next generation sequencing
Two-hundred twenty-five nanograms of genomic DNA extracted from frozen tumor tissues were used to generate NGS libraries. Five 10 μm-thick sections per sample were used to extract DNA using the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen). In the initial series, libraries were generated using HaloPlex technology (Agilent technologies, Santa Clara, CA; following the manufacturer’s protocol). The customized HaloPlex kit included all exons and their flanking regions (Supplemental Table S2). Libraries were sequenced in a MiSeq instrument (Illumina, San Diego, CA) in a paired-end run of 150 bp. The average sequencing coverage across regions was 600x and a coverage >20x was obtained in >98.7% of the target regions.

Libraries of the validation cohort were generated using Access-Array technology (Fluidigm) and Nextera XT procedure (Illumina). Briefly, primers to amplify TP53 (exons 4-10), MYD88 (exons 2-8), NOTCH2 (exon 34), CCND3 (exon 5), SGK1 (all exons), STAT3 (exons 20-21), STAT6 (exons 11-17), PIM1 (exons 1-14), FBXW7 (all exons) and TMEM30A (exons 1-7) regions were designed with the D3-Assay Design web-based tool (Fluidigm) (Supplemental Table S14). Libraries were generated using
50 ng of total DNA in the Access-Array system with a BioMark thermal cycler (Fluidigm) and sequenced in a MiSeq instrument with a paired-end run of 210 bp. Specific primers to sequence NOTCH1 (exon 34), SOCS1 (all exons) and FOXO1 (all exons) were designed using the Primer3 program (Supplemental Table S14). Long-PCR amplifications were performed using the KAPA HiFi DNA Polymerase HotStart ReadyMix (Kapa Biosystems) and normalized with the SequalPrep Normalization Plate kit (Invitrogen). Libraries were generated with the Nextera XT DNA Library Preparation Kit (Illumina) and sequenced with a 2x150 bp MiSeq run. The median sequencing coverage across region was 555x (range 131-933) and a coverage >20x was obtained in >98.7% of the target regions.

**Variant calling algorithms and verification assessment**

Two different bioinformatics pipelines were used for the alignment and variant calling, HD Genome One Research Edition software (DREAMgenics; [http://www.dreamgenics.com/](http://www.dreamgenics.com/)) and Agilent SureCall tool ([http://www.genomics.agilent.com](http://www.genomics.agilent.com)). FASTQ files generated by MiSeq control software were processed using the above mentioned algorithms and both were compared.

### Genome One Research Edition software

A new algorithm was developed in cooperation with DREAMgenics.Inc to perform the alignment, calling and annotation of the variants obtained with Haloplex Libraries. The allelic frequency cut-off for considering mutations was 5%. All variants were confirmed by visual inspection. Low coverage calls (total read depth < 10, or mutated allele count <5 calls) and 17 low quality calls were excluded. We excluded for further analysis all synonymous and intron variants and known polymorphisms included in dbSNP database (dbSNP138), ESP6500 ([http://evs.gs.washington.edu/EVS](http://evs.gs.washington.edu/EVS)) with more than 1% frequency in European population or in our own database of polymorphisms in Spanish population.34

### Agilent SureCall

We used SureCall tool (ver1.1) with all default settings to analyze sequencing results and to call the variants. All variants were confirmed by visual inspections. All
synonymous variants, intron variants and known polymorphisms included in dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) or in our own database of polymorphisms in Spanish population were excluded.  

Variant calls of both algorithms were integrated and, finally, 1331 calls were selected in the 150 cases (Supplemental Figure S2 and Supplemental Table S3). To determine the accuracy of the sequencing method and analytical algorithms, we selected 152 (11%) variants from these 1331 called variants, and we verified them using Sanger sequencing. One hundred fifty-one (99%) of these variants were confirmed, indicating the accuracy of the analysis.

For the validation series, the complete bioinformatic analysis, alignment and variant calling were performed with the MiSeq Reporter Software (MSR, version 2.4.60). All variants detected by any of these two algorithms were combined and annotated using ANNOVAR as well as custom scripts. Like in the initial series analysis, we excluded all synonymous and intron variants and known polymorphisms included in dbSNP database (dbSNP138), ESP6500 (http://evs.gs.washington.edu/EVS/) with more than 1% frequency in European population or in our own database of polymorphisms in Spanish population.

Sanger sequencing. Verification of NGS results and mutational analysis of CDKN2A and 3’UTR region of NOTCH1

Sanger sequencing was used to verify the results of the NGS described above and to analyze Exon 1alpha, 1beta and 2 of CDKN2A and 3’UTR region of NOTCH1. PCR primers were designed using Primer 3 (http://frodo.wi.mit.edu/primer3) and purchased from Sigma-Aldrich (St. Louis, MO). Amplification by PCR was performed using AmpliTaq Gold DNA Polymerase (Life technologies, Grand Island, NY) or QIAGEN Multiplex PCR Kit (Qiagen, Madrid, Spain) with 50ng of DNA and 200µM dNTP mix (Life technologies) following the manufacturer’s recommendations. All PCR products were run in a capillary electrophoresis gel (QIAxcel Advanced System, Qiagen) with the QIAxcel DNA screening kit (Qiagen). The multiband PCR products were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Bethlehem, PA).
Regarding Sanger sequencing, PCR products were cleaned using ExoSAP-IT (Affymetrix, Santa Clara, CA) and sequenced using ABI Prism BigDye terminator v3.1 (Life technologies) with 5 pmol of each primer. Sequencing reactions were run on an ABI-3730 automated sequencer (Life Technologies). All sequences were examined with the Mutation Surveyor DNA Variant Analysis Software (SoftGenetics, State College, PA). Sequences of the used primers are listed in Supplemental Table S14.

Selection of potential driver mutations and verification in germline DNA
Potential driver mutations were selected according to the following criteria (Supplementary Fig S2): 1) We initially selected 216 “relevant” mutations by manual curation based on previous reports and COSMIC, including somatic and functional mutations and mutations clustering in known functional domains.\(^1\, 3, 5, 7, 11, 12, 15, 17, 19, 22, 25, 27, 29-31, 36-54\) 2) All truncating mutations (n= 274), except two found in known oncogenes (\(MYD88\), M11 and \(CD79A\), Q222*), were also considered as potential driver events. 3) The potential drivers of the remaining missense and in-frame mutations were selected based on the functional prediction established by the OncodriveCLUST, Mutation Assessor (MA) and SIFT algorithm.\(^55-57\) MA and SIFT algorithms were selected after comparing several methods including these two and CHASM,\(^58\) CONDEL,\(^59\) FATHMM,\(^60\) Mutation Tester,\(^61\) and Polyphen2 (PPH2).\(^62\) To select the most appropriate algorithm we initially explored the performance of each of these algorithms when distinguishing the variants found in our study that were known polymorphisms or known somatic recurrent mutations described in COSMIC. In this particular data set, the scores that better predicted the expected characteristics of the variant were MA and SIFT, followed by CHASM. We did not observe any benefit in combining these scores. We selected MA because it showed a narrower score range for polymorphisms and larger differences between recurrent and non-recurrent COSMIC entries as compared to SIFT. For those cases in which the MA score could not be retrieved, we used a SIFT score. Using these two algorithms, 271 out of the 841 missense or in-frame mutations were selected as driver mutations.

To test the accuracy of our "functional prediction" algorithm for missense mutations, we selected 92 variants in 32 patients who had germline DNA available. We observed that
90% of the mutations classified as functional were somatic (28/31) while 89% of the germline mutations were classified as non-functional (24/27) (Supplemental Methods and Supplemental Table S15). The 34 somatic variants predicted as non-functional by the algorithm were not considered drivers. Taking these three criteria together we selected 761 potential driver mutations (58% of the total) for the clinicopathological analysis (Supplemental Figure S2 and Supplemental Table S4).

**Copy Number and Structural Alteration Analysis**

Samples were analyzed using CytoScan HD Array (Affymetrix) according to the manufacturer's instructions. Scanned data from CytoScan HD were processed by Chromosome Analysis Suite (Affymetrix) for subsequent analyses. The analytical programs of “Nexus CN 7.5 Discovery edition” (Biodiscovery, Hawthorne, CA), SNP-FASST2 (Biodiscovery) and ASCAT (http://heim.ifi.uio.no/bioinf/Projects/ASCAT/) were used to analyze genomic alterations. Minimal common regions (MCRs) of gains and losses were picked up using an R custom script. The most frequently altered regions (>=20%) were extracted and selected by visual inspection of two different observers (K.K. and I.S.). A total number of 34 MCRs were selected. Then ABC and GCB type DLBCL cases were analyzed separately and MCRs specific for each molecular subtype were identified.

Genomic alterations satisfying the following criteria were regarded as “deep losses” or “high gains”: (1) An amplitude of an alteration was more than 1 or less than -1 in log2 ratio. (2) Deeper and shorter gains or losses were identified in longer and shallower alterations (gain in gain or loss in loss). “Homozygous loss” and “amplification” were defined as “deep loss” and “high gain” regions less than 5Mb, respectively. 6q14.1 and 10q23.31 regions, in which TMEM30A and PTEN were located respectively, were further added to the alteration list because they showed recurrent homozygous losses. Additionally, 17 regions previously reported were included. In total, 62 chromosomal regions were selected for further analysis including clinical correlation. Loss of heterozygosity (LOH) without genomic alteration (CNN-LOH) was considered when the size of the altered region was >5Mb. Cases were regarded to have
chromothripsis when at least seven switches between two or more copy number states were detected on an individual chromosome in which LOH was retained.71

The copy number alterations of CDKN2A were investigated in the validations series using TaqMan® Genotyping Master Mix and TaqMan® Copy Number Assay Hs02738179_cn for CDKN2A. DNA was analyzed using duplicates in a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). Relative quantification of the gene locus was analyzed with the 2^ΔΔCt method using TaqMan® Copy Number Reference Assay RNaseP as the endogenous control and JVM-2 cell line (wild type for CDKN2A) as mathematical calibrator. We included the cell line MAVER-1 as a control of homozygous deletion.

Interphase fluorescence in situ hybridization (FISH) analysis was carried out on FFPE sections to detect MYC, BCL2 and BCL6 breaks using specific probes supplied by Abbott Molecular (Des Plaines, IL, USA) following the manufacturer’s specifications. The FISH probe used for the BCL6 FISH analysis was the LSI BCL6 (ABR) Break Apart Rearrangement Probe, (Abbot Molecular Des Plaines, USA) that identifies both breakpoints located in the major and alternative breakpoint region.

**Cell of origin determination**

The molecular cell of origin (COO) of the tumors was established using the Gene Chip Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) and/or the Lymph2Cx assay (Nanostring technologies, Seattle, WA). To determine the COO in the training series, total RNA was extracted from frozen tissues using RNeasy Kit (Qiagen) following the manufacturer's instructions. For the validation series, total RNA was obtained from formalin-fixed paraffin-embedded material (FFPE-M). Five 10 μm-thick sections per sample were used to extract RNA using the RNeasy FFPE Kit according to the manufacturer's instructions (Qiagen). RNA integrity from frozen specimens was examined with the Agilent 2100 Bioanalyzer (Agilent Technologies) and only high-quality RNA samples were hybridized to Affymetrix Human Genome Array U219 array plates according to Affymetrix standard protocols. Summarized expression values were computed using the robust multichip average approach
implemented in the Expression Console Software (Affymetrix Inc.). COO was
determined as previously reported.\textsuperscript{73} For RNA extracted from FFPE tissues we used a
digital multiplexed gene expression analysis with the nCounter/Nanostring technology
following the established protocol.\textsuperscript{72} Samples were classified as GCB, ABC and
Unclassified (UC) subtypes using the algorithm previously described.\textsuperscript{72}

**Gene expression analysis**
To verify the biological relevance of NOTCH pathway activation in DLBCL, we
compared the gene expression profiles of 12 cases with NOTCH pathway mutations (5
*NOTCH2*, 4 *SGK1*, 2 *NOTCH1*, 1 *FBWX7*) and 27 with wild-type genes of this pathway
using Affymetrix® Human Genome U219. A gene set enrichment analysis (GSEA) was
performed comparing *SGK1* mutated and unmutated cases. We tested the KEGG
NOTCH signaling pathway, two lists of genes upregulated by NOTCH signaling and
two other gene-sets downregulated by NOTCH.\textsuperscript{74, 75} In addition, *HES1* mRNA
expression was analyzed in 14 cases with NOTCH pathway mutations and 13 with wild-
type genes of this pathway by qRT-PCR using a designed human Taqman® Gene
expression Assay for *HES1* (Hs00172878_m1; Applied Biosystems, Foster City, CA).
Gene expression was quantified by the comparative cycle threshold (Ct) method (\(\Delta\Delta\text{Ct}\))
using GUS as endogenous control. All real-time PCR reactions for the individual
samples were performed in triplicate. Results were expressed as relative gene
expression (versus *GUS* gene expression) using arbitrary units.

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Supplemental Figure 1. PFS and OS in training and validation series.
Total variant calls (n=1331)

Evaluateable mutations (n=1115)

- Missense mutations, in-frame mutations (n=841)
  - Mutation Assessor/SIFT/OncodriveCLUST: Nonfunctional (n=570)
  - Mutation Assessor/SIFT/OncodriveCLUST: Functional (n=271)

- Other truncating mutations (n=274)

Mutations described previously as somatic or functional (n=216)

Potential driver mutations (n=761)
Supplemental Figure 3. Genetic alterations characterized in 150 DLBCL patients.

- Frequently mutated genes (>5%)
- Mutated genes (<5%)
- No mutated genes

Genes: BCL2, BCL6, and MYC rearrangements

Gene set enrichment analysis (GSEA) results.
Supplemental Figure 4. Alterations of four genes in the common deleted 6q14-q23 region.

A

B

Biallelic (Loss + no truncating mutation)
Biallelic (Loss + at least one truncating mutation)
No truncating mutation
Truncating mutation
Homozygous deletion
Only loss
Supplemental Figure 5. Chromothripsis-like patterns detected in DLBCL cases.

A

chr 13

miR17-92

B

REL/BCL11A

chr 2

Amplification

Gain

Loss

Homozygous deletion
Supplemental Figure 6. GSEAs of SGK1 and NOTCH pathway mutated cases vs not mutated.

A

Mutated in SGK1 (4) vs Not mutated in Notch Pathway (27)

NES = 1.27
FDR q-value = 0.319

NES = 1.24
FDR q-value = 0.185*

NES = 1.64
FDR q-value = 0.038*

NES = 1.67
FDR q-value = 0.017*

NES = 1.51
FDR q-value = 0.069*

NES = 1.37
FDR q-value = 0.148*


B

Mutated in NOTCH pathway (12) vs Not mutated in Notch Pathway (27)

NES = -1.4
FDR q-value = 0.154*

NES = -1.72
FDR q-value = 0.022*

NES = -1.65
FDR q-value = 0.037*

NES = -1.48
FDR q-value = 0.067*

NES = -1.48
FDR q-value = 0.067*

Genes up-regulated by Notch signalling pathway

Genes down-regulated by Notch signalling pathway