Targeted next generation sequencing reveals high mutation frequency of CREBBP, BCL2 and KMT2D in high-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangements

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Supplemental data

Supplemental Methods

Patients:

Twenty adult patients diagnosed with DHL or THL who harbor gene rearrangements involving MYC and BCL2 or/and BCL6 determined using the FISH test were selected\(^1\). This cohort includes 15 patients with rearrangements of MYC and BCL2, 2 patients with rearrangements of MYC and BCL6 and 3 patients with rearrangements of MYC, BCL2 and BCL6. The COO of each tumor was assessed using the Hans algorithm\(^2\). 18 patients were classified as GCB-DLBCL and 2 as non-GCB-DLBCL. All patients signed informed consent forms. Clinical, immunohistochemical and cytogenetic features of the DHL/THL patients are summarized in Table 1.

DNA extraction and quantification

Tumor DNA was extracted from a 10µm-thick section of FFPE samples. DNA extraction was performed using the Maxwell® 16 FFPE Plus LEV DNA Purification Kit (Promega) based on the manufacturers’ instructions. Extracted DNA was quantified and qualified for an NGS assay as reported previously by our group\(^3\).

Lymphopanel design

Specific exons or hotspots of 43 clinically relevant genes involved in lymphomagenesis were selected for targeted sequencing based on an extensive literature review of NGS studies in de novo or relapsed/refractory DLBCL\(^4\)–\(^12\). These selected regions are listed in Supplementary Table S1. We used an Illumina TruSeq Custom Amplicon (TSCA dual strand, v1.5 chemistry) assay for the deep sequencing of these regions. The custom mixture of oligonucleotides generated 1224 amplicons of suitable size (mean: 159bp and median: 153bp [150bp-180bp]) covering 124 kb. Samples were prepared following the TSCA protocol and as described elsewhere\(^3\). Multiple indexed libraries were pooled and sequenced on the Illumina MiSeq using a V3 flow cell. After pair-end sequencing (2x150 cycles), the four FastQ files generated per sample by the MiSeq Reporter software (v2.6.2.3, Illumina) were analyzed using Amplicon DS (v1.1.13.0, Illumina). Filter criteria used for variant calling single nucleotide variants (SNVs) and short insertions and deletions (indels) are described below.

Somatic alteration assessment and filtering

In Amplicon DS software, variant scores are computed using a Poisson model that excludes calls with scores below Q20. Variants are first called separately for each pool and are then compared and combined into a single output file. The algorithm only calls variants for bases that are covered at a depth of 300X or greater for a single amplicon. Variants were called if they were present in both libraries with a mean VAF of 5% or greater. Following variant calling, variants detected by AmpliconDS software were filtered regarding their consequence. Variants that were missense variant, frameshift, stop gained, stop lost, initiator codon, in-frame insertion, in-frame deletion and splice-site variant were kept and analyzed. Stop-gain, frameshift and/or splicing SNVs are referred to in the article as truncating mutations. Variants
having a frequency of 1% or more in the population (in database Exome Aggregation Consortium (ExAC) Variants, Variants Exome Sequencing Project (ESP) or 1000 Genomes Project) were considered polymorphisms and were excluded. Alamut visual (v2.8, Interactive-Biosoftware, Rouen, France) or Integrative Genomics Viewer (IGV, v2.3.97, Broad Institute, Cambridge, Massachusetts, USA) were used to look at the alignment when necessary. To validate our Lymphopanel, we first re-sequenced patients #3,4,5, 6 and 7 with the same protocol previously described (Amplicon targeted NGS and Lymphopanel) and found precisely the same variants for each patient with similar allele frequency (Table S3). Secondly, we sequenced these 5 patients by capture hybridization sequencing using a similar panel allowing us to find the same variants (Table S3). Briefly, DNA probe sets corresponding to genomics regions of interest were designed using the NimbleGen proprietary tool. A total of 100ng of DNA was sheared with a Covaris ME220 system to provide average fragments of 200bp. The sample preparation and target enrichment were performed according to the Roche SeqCap EZ Hypercap Workflow (ROCHE). Samples were sequenced on an Illumina MiseqDX system with the Illumina V3 chemistry cartridge reagent. The variants are called with Illumina's Enrichment workflow (v0.0.0.0).

**Comparator cases of DLBCL NOS and GCB-DLBCL**

Comparator cases of DLBCL NOS patients were selected from recent studies of whole exome sequencing or targeted sequencing 4–10,13–17. From these 12 studies of whole exome sequencing or targeted sequencing in DLBCL NOS patients, 7 studies clearly set out their data with the possibility for each case to link its mutational profile to its COO and have been selected as comparator cases of GCB-DLBCL 8–10,13–15,17. Likewise, 3 of 12 studies including patient clinical outcome have been selected to compare the overall survival (OS) of DLBCL NOS to that of 19 DHL/THL patients with clinical outcome available15–17.

**AID mutation analysis**

For AID mutation analysis, synonymous variants were also included, and the filter regarding the consequence of the variants was not applied. Preferential DGYW/WRCH AID target sites were targeted.

**Statistical analysis**

Mutation frequencies in literature were estimated by pooling data from studies of WES or targeted NGS or high-throughput sequencing in DLBCL NOS 4–10,13–17 or in GCB-DLBCL 8–10,13–15,17. Differences in mutation frequencies were assessed using Fisher’s exact test for count data. Differences in mutation pathway frequencies were assessed using the \( \chi^2 \) test. All reported p-values were two-sided. Kaplan Meier survival curves were used for survival analysis. Differences in survival curves were assessed using the log-rank test. For all statistical tests, differences were considered significant at the 5% level. Statistical analyses were conducted using R 3.4.3 software or GraphPad Prism version 5.00 (GraphPad Software).
Supplemental Figure

Figure S1: Overall survival (OS) of DHL/THL patients compared to OS of DLBCL NOS patients
Supplemental Tables

**Table S1: Overview of the lymphopanel used for NGS analysis.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exons/Hotspots</th>
<th>Pathway</th>
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<td>B2M</td>
<td>1 and 2</td>
<td>Immunity</td>
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<td>BCL2</td>
<td>1</td>
<td>Apoptosis/cell cycle</td>
</tr>
<tr>
<td>BCL6</td>
<td>1 to 8</td>
<td>Immunity</td>
</tr>
<tr>
<td>BIRC3</td>
<td>6 to 9</td>
<td>Apoptosis/cell cycle</td>
</tr>
<tr>
<td>BRAF</td>
<td>15</td>
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<td>14 to 16</td>
<td>NFκB</td>
</tr>
<tr>
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<td>4 to 9</td>
<td>NFκB</td>
</tr>
<tr>
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<td>BCR</td>
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<td>CD79B</td>
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<tr>
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<td>CDKN2B</td>
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<td>26, 27, 28 and 34</td>
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<td>JAK/STAT</td>
</tr>
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**Table S2: List of variants detected in DHL/THL patients (VAF: Variant allele frequency)**

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<tr>
<th>Patient #</th>
<th>Gene</th>
<th>Transcript</th>
<th>cDNA alteration</th>
<th>protein alteration</th>
<th>VAF</th>
<th>Total depth</th>
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<td>p.Leu77Ter</td>
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<td>4854</td>
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<td>12</td>
<td>B2M</td>
<td>NM_004048.2</td>
<td>c.128T&gt;G</td>
<td>p.Leu43Arg</td>
<td>42%</td>
<td>4412</td>
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<tr>
<td>1</td>
<td>BCL2</td>
<td>NM_000633.2</td>
<td>c.60T&gt;A</td>
<td>p.His20Gln</td>
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<td>1284</td>
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<td>BCL2</td>
<td>NM_000633.2</td>
<td>c.31A&gt;T</td>
<td>p.Asn11Tyr</td>
<td>25%</td>
<td>1872</td>
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<td>p.Leu121Val</td>
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<td>3</td>
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<td>c.585+4G&gt;A</td>
<td>splice site</td>
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<td>2216</td>
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<td>p.Arg129His</td>
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<td>1196</td>
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<td>p.Pro153Leu</td>
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<td>p.Pro1476Leu</td>
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<td>1034 908 43%</td>
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<td>206 364 45%</td>
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**Table S3**: Validation of the Lymphopanel. (VAF: Variant allele frequency)
Table S4: Comparisons of observed mutation frequencies in DHL/THL and those observed in DLBCL NOS in literature (NA: not available)

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*FOXO1, SOCS1, PKCz, JUN, CREBBP, BCL2, EZH2, MYC, IGLL5, KMT2D, SOCS1, FOXO1, FOXO3A, BIM, RIP1, FOXO1*
Table S5: Comparisons of observed mutation frequencies in GCB-DHL/THL and those observed in GCB-DLBCL in the literature (NA: not available)

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