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

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma, representing approximately 30-40% of all cases.[1] The update of the 4th edition of the 2017 WHO classification provides new concepts in the classification of DLBCL.[1] Importantly, the category "B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt Lymphoma" is being replaced by two new categories: high-grade B-cell lymphoma (HGBL) with *MYC, BCL2* and/or *BCL6* rearrangements so-called double-hit (DH) or triple-hit (TH) lymphomas, and HGBL, not otherwise specified (NOS) without *MYC* and *BCL2* or *BCL6* translocations. Patients with *MYC/BCL2* DHL or *MYC/BCL2/BCL6* THL will usually have an aggressive clinical course despite the fact that most cases have the germinal center B (GCB) cell-of-origin (COO) which is known to be associated with a better prognosis than the activated B-cell (ABC) subtype.[2] Patients with *MYC/BCL6* DHL may have a GCB or ABC COO, and most studies, but not all, reported an aggressive clinical course as well.[2,3] To the best of our knowledge, genomic characterization of a patient cohort diagnosed with *MYC/BCL2* DHL, *MYC/BCL6* DHL or *MYC/BCL2/BCL6* THL has not yet been reported. To identify molecular variants, we performed targeted next-generation sequencing (NGS) of FFPE samples of DHL/THL for mutations on 43 genes known to be important for lymphomagenesis (*Online Supplementary Table S1*) based on an extensive literature review of NGS studies on DLBCL NOS.[4–10]

We thus studied 20 adult patients diagnosed with DHL harboring *MYC* and *BCL2* (n=15) or *MYC* and *BCL6* (n=2) rearrangements and 3 THL. Based on the Hans algorithm, 18 patients were classified as GCB, and 2 as non-GCB.[11] Clinical, immunohistochemical and cytogenetic features of DHL/THLs are summarized in Table 1. All FFPE samples were analyzed and sequenced using our NGS Lymphopanel of 43 genes (see *Online Supplementary Material*).

In all FFPE DHL/THL cases, our NGS Lymphopanel identified 438 alterations on 40 genes important for lymphomagenesis. Of these, 197/438 (45%) alterations localized on 33 genes are non-synonymous and predict amino-acid substitutions or truncation of the proteins (Figure 1A and *Online Supplementary Tables S2-S3*). Missense mutations are the most frequent, 145/197 (74%), followed by 17 (9%) splice-site mutations and 17 (9%) frameshift insertions/deletions. All samples harbor non-synonymous somatic alterations with a minimum of 5 alterations identified in patients #15 and #16 and a maximum of 18 alterations detected in patient #6. The most frequently mutated genes are *CREBBP* (16/20 cases) followed by *BCL2* (12/20), *KMT2D* (12/20), *MYC* (9/20), *EZH2* (8/20), *IGLL5* (8/20), *FOXO1* (6/20) and *SOCS1* (6/20) (Figure 1A). Mutations of these genes have previously been reported in DLBCL NOS, especially in GCB-DLBCL.[4–10,12–16] Interestingly, the percentage of DHL/THL with mutations on these 8 genes is significantly higher than that reported in DLBCL NOS (Figure 1B and *Online Supplementary Table S4*).[4–10,12–16] As most DHL and all THL were classified as GC subtype, we compared the mutation rates between GCB-DLBCL and DHL/THL with GC subtype (GCB-DHL/THL). Figure 1C shows that the percentage of GCB-DHL/THL with *CREBBP, BCL2, KMT2D, MYC, EZH2* and *FOXO1* mutations is significantly higher than that report-

Table 1. Clinical, immunohistochemical and cytogenetic features of DHL/THL patients.

Case #	Age	Sex	Localization	Hans	CD20	CD10	BCL6	IRF4 /MUM1	BCL2	Ki67 (%)	BCL2t	MYCt	BCL6t	EBER
1	81	M	sus clavicular LN	GCB	+	+	ND	–	+	50-60	+	+	–	–
2	53	M	thorax LN	GCB	+	+	ND	ND	+	80	+	+	–	ND
3	63	F	Bronchus	GCB	+	+	+	–	–	85	+	+	–	ND
4	68	F	jejunum wall	GCB	+	+	+	–	+	60-70	+	+	–	–
5	63	M	retroperitoneal biopsy	GCB	ND	+	+	ND	+	95	+	+	–	ND
6	69	F	retroperitoneal LN	GCB	+	–	+	–	–	40-50	+	+	ND	–
7	79	M	retroperitoneal mass	GCB	+	+	+	–	+	40	+	+	–	–
8	83	F	rectum biopsy	GCB	–	+	–	ND	–	90	+	+	–	–
9	83	F	sus clavicular LN	GCB	+	+	+	–	+	80	+	+	–	–
10	43	M	mesenteric LN	GCB	+	+	+	–	+	70	+	+	–	–
11	57	M	inguinal LN	GCB	+	+	+	–	+	70	+	+	–	–
12	67	F	submandibular mass	GCB	+	+	+	–	+	80	+	+	–	ND
13	60	M	lumbosacral	GCB	+	+	+	–	+	80	+	+	–	ND
14	79	M	retroperitoneal	GCB	+	+	ND	–	+	ND	+	+	–	ND
15	53	M	LN	Non-GCB	+	–	–	+	+	80	+	+	–	–
16	91	M	pleural biopsy	GCB	+	–	+	–	–	100	–	+	+	ND
17	59	F	retroperitoneal	Non-GCB	+	–	+	+	+	90	–	+	+	ND
18	71	F	LN	GCB	+	+	–	+	+	50	+	+	+	–
19	68	F	big epiploon biopsy	GCB	+	+	+	–	+	80-90	+	+	+	–
20	64	M	cervical LN	GCB	+	+	+	–	+	70	+	+	+	ND

F: female; M: male; LN: lymph node; GCB: germinal center B-cell subtype according to the Hans algorithm; ND: not determined; BCL2t: translocation of BCL2; MYCt: translocation of MYC; BCL6t: translocation of BCL6; EBER, Epstein-Barr virus encoded small RNA detected by chromogenic *in situ* hybridization; +: positive; -: negative

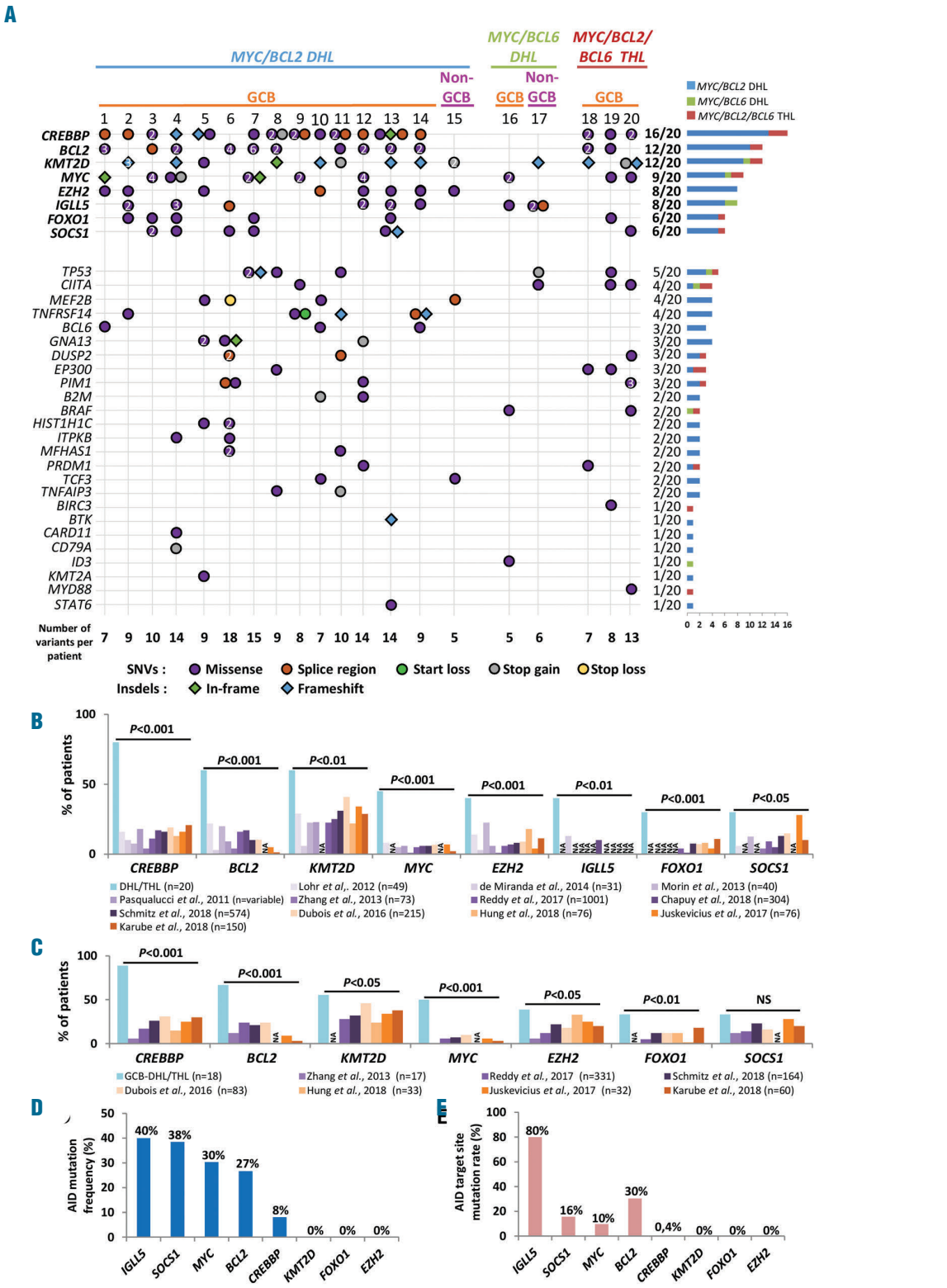

Figure 1. Molecular characterization of DHL. A: Genes mutated in the 20 DHL/THL. Somatic events are reported as colored symbols, with an optional white number signaling multiple events in the same category. Genes are listed in rows on the left and are listed by mutation frequency. The genes in bold are the most frequently mutated genes in DHL/THL. The 20 DHL/THL patients are described in distinct columns, grouped by their double or triple translocations and their COO subtypes. The right panel visually summarizes the number of patients having a mutation for each gene. At the bottom, the total number of variants found for each patient is indicated. B: Frequency of gene mutations in DHL/THL (in blue) compared to frequency of gene mutations in DLBCL NOS described in literature (studies that used WGS or WES are in purple, studies with targeted NGS or high-throughput sequencing are in orange). C: Frequency of gene mutations in GCB-DHL/THL (in blue) compared to frequency of gene mutations in GCB-DLBCL described in the literature (studies that used WGS or WES are in purple, studies with targeted NGS or high-throughput sequencing are in orange). D: AID mutation frequencies (percentage of total variants located at preferential DGYW/WRCH target sites) calculated for genes highly mutated in DHL/THL. E: AID target site mutation rates (percentage of sequenced DGYW/WRCH AID target sites in each gene effectively mutated) calculated for genes highly mutated in DHL/THL. NA: not available.

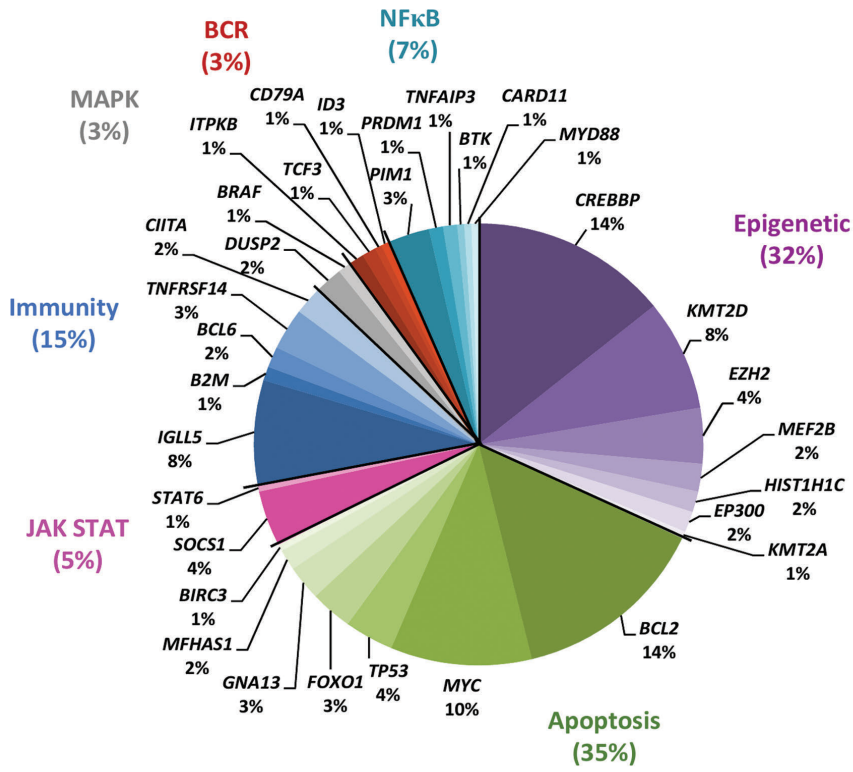

Figure 2. Mutation pathway frequencies. Mutation frequencies per gene are shown as a percentage of the total number of variants.

ed in GCB-DLBCL (P<0.001, P<0.001, P<0.05, P<0.001, P<0.05 and P<0.01 respectively; *Online Supplementary Table S5*). These results suggest that the mutation rate of these 6 genes in DHL/THL is not due only to COO classification.[8–10,12–14,16]

The *CREBBP* gene is frequently mutated in most DHL/THL cases (16/20 cases) (Figure 1A). *CREBBP* mutations are detected in all cases of THL and only in DHL with *MYC* and *BCL2* rearrangements. Twenty-eight somatic alterations are identified: 17 missense mutations, 10 truncating mutations and 1 in-frame deletion. More than 50% of missense mutations and all the truncating mutations are located before and/or in the acetyltransferase HAT domain with predicted loss of protein function. Thus, most of the somatic alterations on *CREBBP* alter its acetyltransferase activity, leading to constitutive activation of the BCL6 oncoprotein and to a decrease in p53 tumor suppressor activity.

In addition, the *BCL2* gene is frequently and highly mutated (28 mutations) in our cohort (12/20 cases), notably in *MYC* and *BCL2* rearranged DHL (12/18 patients) (Figure 1A-C). Similarly, numerous mutations of MYC (20 mutations) are found in 9/20 DHL/THL and mostly in *MYC* and *BCL2* translocated DHL. These results are consistent with previous studies on DLBCL NOS with isolated translocations showing a strong positive correlation of *MYC* and *BCL2* mutations and their respective translocations.[17]

As described in most GCB-DLBCL, DHL/THL harbor mutations on the *KMT2D* (12/20) and *EZH2* (8/20) genes. Intriguingly, the frequencies of *KMT2D* and *EZH2* mutations are significantly higher in DHL/THL compared to both DLBCL NOS (Figure 1B) (P<0.01 and P<0.0001, respectively) and the GCB subtype (Figure 1C) (P<0.05). *KMT2D* mutations are detected in both *MYC/BCL2* and *MYC/BCL6* DHLs and in THLs and are mostly frameshift deletions or stop gain alterations leading to a loss of protein function. All 8 EZH2 mutations are only found in *MYC/BCL2* DHL and 6/8 are hotspot Y646 mutations.

Mutations of *FOXO1* found in 6/20 cases are exclusively detected in patients with MYC and BCL2 rearrangements (DHL or THL) (Figure 1 A-C). Among them, 5 *FOXO1* mutations are found within the first exon of *FOXO1* encoding the entire N-terminal region and part of the Forkhead box domain. All 5 mutations are hotspot mutations: R19 (n=2), R21 (n=1) and T24 (n=2), previously described in DLBCL NOS.[18] *SOCS1* mutations are only found in *MYC* and *BCL2* rearranged DHL (6/20). The 8 mutations of the *SOCS1* gene detected spread throughout the coding region and among them, 7 are non-truncating mutations (Figure 1A-C). Furthermore, 40% of DHL/THL (n=8/20) display at least one mutation on *IGLL5* known to be recurrently mutated in DLBCL.[7] Most alterations detected on *IGLL5* are missense mutations (14/16). Moreover, 40% of synonymous and non-synonymous mutations on *IGLL5* are located at DGYW/WRCH target sites, suggestive of activation-induced cytidine deaminase (AID) activity.

We thus assessed the impact of AID involvement on the lymphopanel genes. To address this question, we looked at the percentage of mutations potentially induced by aberrant somatic hypermutation (SHM) occurring within the DGYW/WRCH DNA sequence motifs. Among the highly mutated genes, *IGLL5, SOCS1, MYC* and *BCL2* have the highest AID mutation frequency (40%, 38%, 30% and 27%, respectively) whereas *KMT2D, FOXO1* and *EZH2* show no AID induced mutations (Figure 1D). Moreover, the percentage of sequenced DGYW/WRCH AID target sites mutated on these genes is also higher on *IGLL5, SOCS1, MYC* and *BCL2* whereas *CREBBP* shows a very low AID target site mutation rate compared to its AID mutation frequency (0.4% *vs.* 8% of mutation rates) (Figure 1E).

We then studied the functional significance of these DHL/THL genetic alterations using 8 predefined signaling pathways (*Online Supplementary Table S1*). As shown in Figure 2, DHL/THL mutations are mainly involved in apoptosis/cell-cycle (35% of total variants) and epigenetic regulation pathways (32% of total variants mostly occurring on the *CREBBP* gene), known to be associated with a poor prognosis in lymphoma patients.[9,13] Interestingly, the number of alterations in the apoptosis/cell cycle pathway is significantly higher in DHL/THL than in DLBCL NOS and GCB-DLBCL according to the Dubois *et al*. study, (70/197 *vs*. 209/1064; *P*<0.001 and 42/87 *vs*. 118/450; *P*<0.001, respectively).[9] In contrast, mutations on exon 34 of *NOTCH1* and *NOTCH2* have been reported in 9% of DLBCL NOS cases while the sequencing of the same region showed no mutation in all DHL/THL cases.[13] In addition to *NOTCH1* and *NOTCH2* genes, further analyses of other genes involved in *NOTCH* signaling are needed to study the genomic alterations of this pathway in DHL/THL.

To our knowledge, our data provide a comprehensive profile of genomic alterations of DHL/THL in which the *CREBBP* gene appears to be the most frequently mutated. Based on an extensive literature review of targeted-NGS studies in DLBCL, we were able to datamine only 7 DHL/THL cases from the Karube *et al*. study.[13] They also showed a high mutation rate of *CREBBP* in 6 DHL/THL cases, which exclusively harbored *MYC/BCL2* translocations. These results extend those that we obtained in our cohort. Furthermore, our lymphopanel highlights the high frequency of mutations on *CREBBP* and *FOXO1* known to be associated with a poor prognosis in DLBCL patients illustrated by the significantly worse overall survival (OS) of DHL/THL patients as compared to the OS of DLBCL NOS patients from 3 different cohorts (*P*<0.0001) (*Online Supplementary Figure S1*). We also found a high frequency of *SOCS1* non-truncating mutations which have been associated with a poor outcome in DLBCL patients, but needs to be confirmed in larger cohorts.[10,19]

In conclusion, our study describes for the first time the mutational landscape of DHL/THL, a poorly studied subtype of aggressive B-cell lymphoma. Using FFPE samples of 20 DHL/THL cases, we identified numerous mutations on genes involved in apoptosis/cell-cycle and epigenetic regulation pathways. Our lymphopanel of 43 genes identifies a distinct mutational signature of DHL/THL with a higher a level of *CREBBP, BCL2, KMT2D, MYC, EZH2* and *FOXO1* mutations than in GCB DLBCL-NOS. Finally, our findings may provide a rationale for therapeutic strategies in patients with the most clinically aggressive DLBCL subgroup.

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