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ORIGINAL ARTICLE

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

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Genome studies of diffuse large B-cell lymphoma (DLBCL) have revealed a large number of somatic mutations and structural alterations. However, the clinical significance of these alterations is still not well defined. In this study, we have integrated the analysis of targeted next-generation sequencing of 106 genes and genomic copy number alterations (CNA) in 150 DLBCL. The clinically significant findings were validated in an independent cohort of 111 patients. Germinal center B-cell and activated B-cell DLBCL had a differential profile of mutations, altered pathogenic pathways and CNA. Mutations in genes of the NOTCH pathway and tumor suppressor genes (*TP53/CDKN2A*), but not individual genes, conferred an unfavorable prognosis, confirmed in the independent validation cohort. A gene expression profiling analysis showed that tumors with NOTCH pathway mutations had a significant modulation of downstream target genes, emphasizing the relevance of this pathway in DLBCL. An *in silico* drug discovery analysis recognized 69 (46%) cases carrying at least one genomic alteration considered a potential target of drug response according to early clinical trials or preclinical assays in DLBCL or other lymphomas. In conclusion, this study identifies relevant pathways and mutated genes in DLBCL and recognizes potential targets for new intervention strategies.

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is a highly heterogeneous neoplasm.[1] Although current therapies have improved the clinical outcome, 30–40% of the patients are still not cured.[2] Understanding the molecular basis of this heterogeneity may facilitate the design of alternative management strategies including specific targeted therapies. The cell of origin (COO) of these tumors, germinal center B-cell (GCB) or activated B-cell (ABC), is one of the major sources of diversity associated with different molecular alterations and clinical evolution.[3–9] More recently, next-generation sequencing (NGS) studies have provided a comprehensive catalog of somatic mutations in DLBCL that may also contribute to their heterogeneous behavior.[10–13] However, the number of patients analyzed is still relatively small and the clinical significance of these new mutations remains unknown. On the other hand, few NGS mutational studies have compared the mutational profile of the tumors with their respective chromosomal alterations.[14] Therefore, an integrative view of these two layers of genomic information may provide a better understanding of their influence on the behavior of DLBCL.

One of the major goals of large-scale genomic analyzes of tumors is to identify new targets for therapeutic intervention. However, these comprehensive studies are confronted with the challenge of identifying appropriate candidate drugs for individual patients from the increasing catalog of available drugs that could be tested in new preclinical and clinical studies. The fulfillment of this major objective of precision oncology may require the assistance of bioinformatics tools that integrate the personalized genomic profiles of the tumors with the vast information of potential available drugs.[15,16]

The goal of this study was to determine the clinical relevance of recurrent genomic alterations of DLBCL and their potential value in the management of patients. We have performed an integrated analysis of genomic alterations and mutations in a large panel of genes in DLBCL and run an *in silico* prescription strategy that connects the individual genomic profile with druggability options.[16]

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PATIENTS AND METHODS

Patients and samples

One hundred fifty patients diagnosed with *de novo* DLBCL, not otherwise specified (NOS),[1] from 2002 to 2014, including 14 primary extranodal cases, were selected for this study. Primary mediastinal large B-cell lymphomas and other DLBCL subtypes were excluded. Cases were selected based on the availability of high quality DNA obtained from frozen tissue samples with high tumor cell content (>60%). In the same period of time, 403 patients with DLBCL-NOS were not studied due to the lack of adequate material. These patients had similar clinical features to those of the included patients (Supplementary Table 1). The tumor COO, GCB, ABC or unclassified (UC), was established using U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) and/or the Lymph2Cx assay (NanoString technologies, Seattle, WA, USA).[17] The patients' main clinical features and outcome are detailed in Supplementary Figure 1 and Table 1. Most patients (126, 85%) were treated with a median of 6 courses (range, 1–6) of R-CHOP (rituximab, cyclophosphamide, adriamycin, vincristine and prednisone) and the remainder with regimens without adriamycin mainly due to their age or previous heart disease. Only patients receiving R-CHOP were included in the prognostic analyzes.

A validation series of 111 patients (54M/57 F; median age 63 years) diagnosed over the same period of time was selected from different Spanish and Japanese institutions (Table 1). Ninety patients (86%) were treated with immunochemotherapy, including adriamycin-containing regimens and only these were included in prognostic studies. Patients in the initial and validation cohorts had similar features and outcome (Table 1 and Supplementary Figure 1). This study was approved by the Institutional Review Board of Hospital Clínic (Barcelona, Spain). Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Targeted next-generation sequencing and mutational analysis

We performed targeted NGS of 106 genes selected from previous DLBCL genome sequencing studies (Supplementary Table 2 and Supplementary Methods).[10–13] Libraries were generated using HaloPlex (Agilent technologies, Santa Clara, CA, USA) and sequenced in a MiSeq instrument (Illumina, San Diego, CA, USA). Sequencing data have been deposited at the European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena) under accession number ERP021212. In addition, exon1α, 1β and 2 of *CDKN2A* and the 3'UTR region of *NOTCH1* were analyzed by Sanger sequencing (Supplementary Methods). Two different bioinformatics pipelines (DreamGenics and SureCall tools) were used for the alignment and variant calling (Supplementary Methods). Combination of the two algorithms identified 1331 variant calls (Supplementary Table 3). The accuracy of the calls was confirmed by verifying 99% (151/152) of the selected variants by Sanger sequencing (Supplementary Methods). A selection of driver mutations with potential functional effect was performed based on the criteria described in Supplementary Methods and Supplementary Figure 2. Briefly, potential driver mutations included: (1) 'truncating mutations' ($n=274$), (2) 'relevant mutations' manually curated based on previous reports in the literature and COSMIC database. This group included somatic and functional mutations and mutations clustering in known functional domains ($n=216$), and (3) missense mutations identified as 'functional mutations' by the Mutation Assessor (MA), OncodriveCLUST and SIFT algorithms ($n=271$).[18] 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) (Supplementary Methods and Supplementary Table S15). Taking these three criteria together, we selected 761 potential driver mutations for the clinicopathological analysis (Supplementary Table 4). Virtually all identified mutations (96.3%) showed allelic frequencies ⩾10%.

Thirteen mutated genes with significant clinical impact in the initial series were selected for validation in the independent cohort of patients. Libraries of these genes were generated using the Access-Array system (Fluidigm, South San Francisco, CA, USA) and Nextera XT (Illumina), sequenced and analyzed as described (Supplementary Methods).

Table 1. Initial features and outcome of patients with DLBCL of the initial and validation series

Parameter	*Initial (N = 150)*	*Validation (N = 111)*
Age >60 years	93/149 (62%)	73/109 (67%)
Male gender	78/150 (52%)	54/111 (49%)
Stage III/IV	**78/148 (53%)**	**71/108 (67%)***
ECOG 2 or higher	56/145 (39%)	36/101 (37%)
Extranodal involvement	73/148 (49%)	51/108 (47%)
Bone marrow involvement	**14/150 (9%)**	**21/111 (19%)***
High serum LDH	**63/141 (45%)**	**67/109 (62%)***
High serum B2m	71/125 (57%)	25/46 (54%)
R-IPI score		
Very good	18/148 (12%)	10/110 (9%)
Good	70/148 (47%)	44/110 (40%)
Poor	60/148 (41%)	56/110 (51%)
Cell of origin		
GCB	60/122 (49%)	41/71 (57%)
ABC	55/122 (45%)	21/71 (30%)
UC	7/122 (6%)	9/71 (13%)
Adriamycin-containing treatment	126/148 (85%)	91/106 (86%)
CR rate[a]	92/126 (73%)	63/86 (73%)
5-year PFS[a]	54%	53%
5-year OS[a]	61%	68%

Abbreviations: ABC, activated B-cell; CR, complete response; GCB, germinal center B-cell; OS, overall survival; PFS, progression-free survival; UC, unclassified. *$P<0.05$. Entries in bold mean the differences are statistically significant. [a]Only patients treated with R-CHOP.

Copy number analysis

DNA copy number alterations (CNA) were examined in 119 cases using Cytoscan HD arrays (Affymetrix) and analyzed using Nexus CN 7.5 Discovery edition (Biodiscovery, Hawthorne, CA, USA) as described.[19] Minimal common regions of gain and loss, and copy number neutral loss of heterozygosity (CNN-LOH) were defined as described in Supplementary Methods. Deletions of *CDKN2A* locus were examined by quantitative PCR in the validation series (Supplementary Methods). Copy number data have been deposited at GEO database under accession number GSE94705.

Statistical methods

Complete response (CR), progression-free survival (PFS) and overall survival (OS) definitions were the standard ones.[20] χ^2 method was used for categorical variables and Student's *t*-test for continuous variables. Non-parametric tests were applied when necessary. Actuarial survival analysis was performed by the Kaplan–Meier method and differences assessed by the log-rank test. Multivariate Cox regression analysis was used to assess the independent prognostic impact of different variables in terms of PFS and OS. The *P*-values for multiple comparisons were adjusted using the Benjamini–Hochberg correction. Statistical analyzes were carried out with SPSS v.22 and R software v3.1.3.

In silico drug prescription

Genomic-guided potential therapeutic opportunities for each DLBCL patient were identified *in silico* by using the Cancer Genome Interpreter modified from our previous described pipeline[16] (https://www.cancergenomeinterpreter.org/). The platform matches the genomic alterations of a tumor with an expert manually curated database of genomic alterations that can be used as biomarkers of drug sensitivity, resistance and severe toxicity. The biomarkers database is organized according to the level of clinical evidence supporting the genotype–phenotype association including clinical guidelines, late (phases III–IV) or early clinical trials (phases I–II), case reports and preclinical studies.[21] The biomarkers are classified as: (1) 'Biomarker and tumor match' for those alterations reported to be targets of specific drugs in DLBCL or other lymphoid neoplasms; (2) 'Biomarker match of different gene mutation' for those alterations reported to confer sensitivity to a given drug in DLBCL upon other amino acid changes and (3) 'Biomarker match and tumor repurposing' for those genomic alterations described as biomarkers of drug response in other cancers.[15]

RESULTS

Mutational profile of DLBCL

A total of 761 potential driver mutations were identified in 89 out of the 106 genes with a similar number in GCB and ABC-DLBCL (4.8 ± 2.8 vs 4.0 ± 2.4 mutated genes per case, respectively) (Supplementary Table 4 and Supplementary Figure 3). The most frequently mutated genes were *KMT2D*, *MYD88, CREBBP* and *TP53* found in more than 15% of cases whereas 27 additional genes were mutated in more than 5% of patients (Figure 1a). As expected, some genes carried mutations with the imprint of the somatic hypermutational machinery (*BCL6, IRF4, IRF8, CIITA, PIM1, MYC, SOCS1, BCL7A, BTG1* and *BTG2*).[22,23] *MYD88*, *PIM1, CD79B* and *PRDM1* were significantly more frequently mutated in ABC-DLBCL whereas *KMT2D*, *CREBBP*, *TNFRSF14*, *B2M*, *EZH2*, *GNA13*, *FOXO1*, *ACTB* and *SOCS1* mutations were more common in GCB-DLBCL (Figure 1a, Supplementary Table 5). Interestingly, *MYD88* L265P mutation was almost exclusively identified in ABC-DLBCL while non-L265P mutations were also seen in GCB or UC-DLBCL (Figure 1b).[24] *TP53* truncating and missense mutations on the DNA binding domain (DBD) were preferentially found in ABC-DLBCL, whereas other mutations in the gene were equally distributed in both DLBCL subtypes (Figure 1b).

To determine the possible interactions between mutated genes, we evaluated their patterns of association in the same tumors and within predefined pathogenic pathways (Figures 1c and d, Supplementary Table 6). *MYD88* and *CD79B* mutations were significantly concurrent in the same tumors, particularly in ABC-DLBCL (FDR < 0.01), whereas *KMT2D* mutations were associated with *EZH2* and *CREBBP* mutations in GCB (FDR < 0.05). Mutations in other epigenetic regulatory genes (*MEF2B*, *ARID1A* and *EP300*) were frequently seen in the same tumors but intriguingly, *TET2* mutations never overlapped with mutations in other epigenetic genes. Mutations in genes of the B-cell receptor (BCR) signaling (*CD79B*, *CARD11*, *BCL10*, *CD79A*, *BTK, PRKCB* and *MALT1*) tended to occur in different cases although without statistical significance, probably due to the low number of cases for each gene (Figure 1c). Mutations in the PI3K/AKT/mTOR and JAK/STAT pathways were more frequent in GCB-DLBCL, whereas gene aberrations (mutations/deletions) in tumor suppressor genes (*TP53, CDNK2A*) were more represented in ABC-DLBCL ($P < 0.01$) (Figure 1a).

We also observed mutations in genes of NOTCH pathway (*NOTCH2*, *NOTCH1* and *FBWX7*) (Figure 1d). *SGK1* has been suggested to be a negative regulator of NOTCH signaling enhancing NOTCH protein degradation and reducing its activation by the gamma-secretase but its potential role in lymphoid neoplasms has not been explored.[25,26] *SGK1* mutations in our cases were frequently truncating and in some cases associated with loss of the wild-type allele suggesting that they may enhance NOTCH1 activity (Supplementary Table S4). On the other hand, *NOTCH2* and *SGK1* mutations were mutually exclusive (FDR < 0.05) (Figure 1d). To evaluate whether *SGK1* mutations could be considered in the NOTCH pathway in these tumors, we performed a gene-set enrichment analysis (GSEA) comparing *SGK1* mutated and unmutated cases. *SGK1* mutated DLBCL had a significant overexpression of genes upregulated by NOTCH activation in lymphoid cells and a concordant downregulation of gene signatures inhibited by NOTCH (Supplementary Figure 6A).[27,28] Based on these results we evaluated the relevance of NOTCH pathway in DLBCL comparing the gene expression profiling of 12 cases with NOTCH pathway mutations (5 *NOTCH2*, 4 *SGK1*, 2 *NOTCH1* and 1 *FBWX7*) and 27 wild-type tumors (Supplementary Material). The GSEA found a significant overexpression of downstream signaling genes in cases with NOTCH pathway mutations compared with wild-type tumors (Figure 1e and Supplementary Figure 6B). In addition, a qRT-PCR analysis of *HES1* expression showed significantly higher mRNA levels in cases with NOTCH pathway mutations (Figure 1f). All these findings are consistent with the downstream activation of NOTCH signaling in DLBCL with mutations in genes of this pathway.

Copy number and structural alterations

All tumors examined carried CNAs including 1226 losses, 56 homozygous deletions, 1112 gains, 96 amplifications and 270 regions of recurrent CNN-LOH (Supplementary Table 7). The profile of CNA and target genes in ABC and GCB-DLBCL were similar to those previously described (Figures 2a and b, Supplementary Methods and Supplementary Table 8). However, new alterations and potential target genes in the minimal common deleted regions were identified, including losses of *TMEM30A* (6q14.1) (39/119 33%, two homozygous) and *EBF1* (5q33.3) (9/119, 8%, one homozygous) (Figure 2a). Additional homozygous deletions targeted *CDKN2A* ($n = 9$), *CD70* ($n = 2$), *PTEN* ($n = 2$), *CD58* ($n = 1$) and *TNFAIP3* ($n = 1$). Recurrent amplifications included *REL/BCL11A* (11 and 9 cases, respectively), miR17-92 cluster ($n = 7$), *CDK6* ($n = 6$) and *CDK14* ($n = 6$) (Supplementary Table 8).

Integrative analysis of mutations and CNA identified 16 genes with biallelic inactivation including homozygous deletions or heterozygous deletions with concomitant truncating mutations (Figure 2c). As expected, 74% of *TP53* mutations were associated with 17p losses or CNN-LOH. The common deleted 6q14–q23 region included *PRDM1* and *TNFAIP3* (Supplementary Figure 4) but we also identified *SGK1* and *TMEM30A* as novel targets with biallelic inactivation (Figure 2c, Supplementary Figure 4). *MYD88* was the only gene with known homozygous activating mutations in two cases due to CNN-LOH.

We detected chromothripsis-like patterns in 28 (24%) DLBCLs with similar distribution in ABC and GCB-DLBCL. These cases showed more *TP53* aberrations (61 vs 29% $P = 0.004$) and 11q23–q25 gains/amplifications, including *ETS1* and *FLI1,* (57 vs 19%, $P < 0.001$) than cases without chromothripsis. The most affected chromosomes were 13 ($n = 6$), 2 ($n = 5$) and 6 ($n = 5$). Interestingly, regions targeted by chromothripsis included amplifications of *miR17-92* (13q31.3) ($n = 3$) and *BCL11A/REL* (2p16) ($n = 1$) (Supplementary Figures 5A and B).

BCL2, *BCL6* and *MYC* were rearranged in 19% (25/131), 20% (25/122) and 9% (11/124) of the cases, respectively, (Supplementary Figure 3). *BCL2* and *MYC* translocations predominated in GCB-DLBCL whereas *BCL6* translocations were equally distributed in GCB and ABC-DLBCL. Seven cases had a double hit, 6 *MYC/BCL2* in 4 GCB and 2 UC, and one ABC had a *MYC/BCL6*.

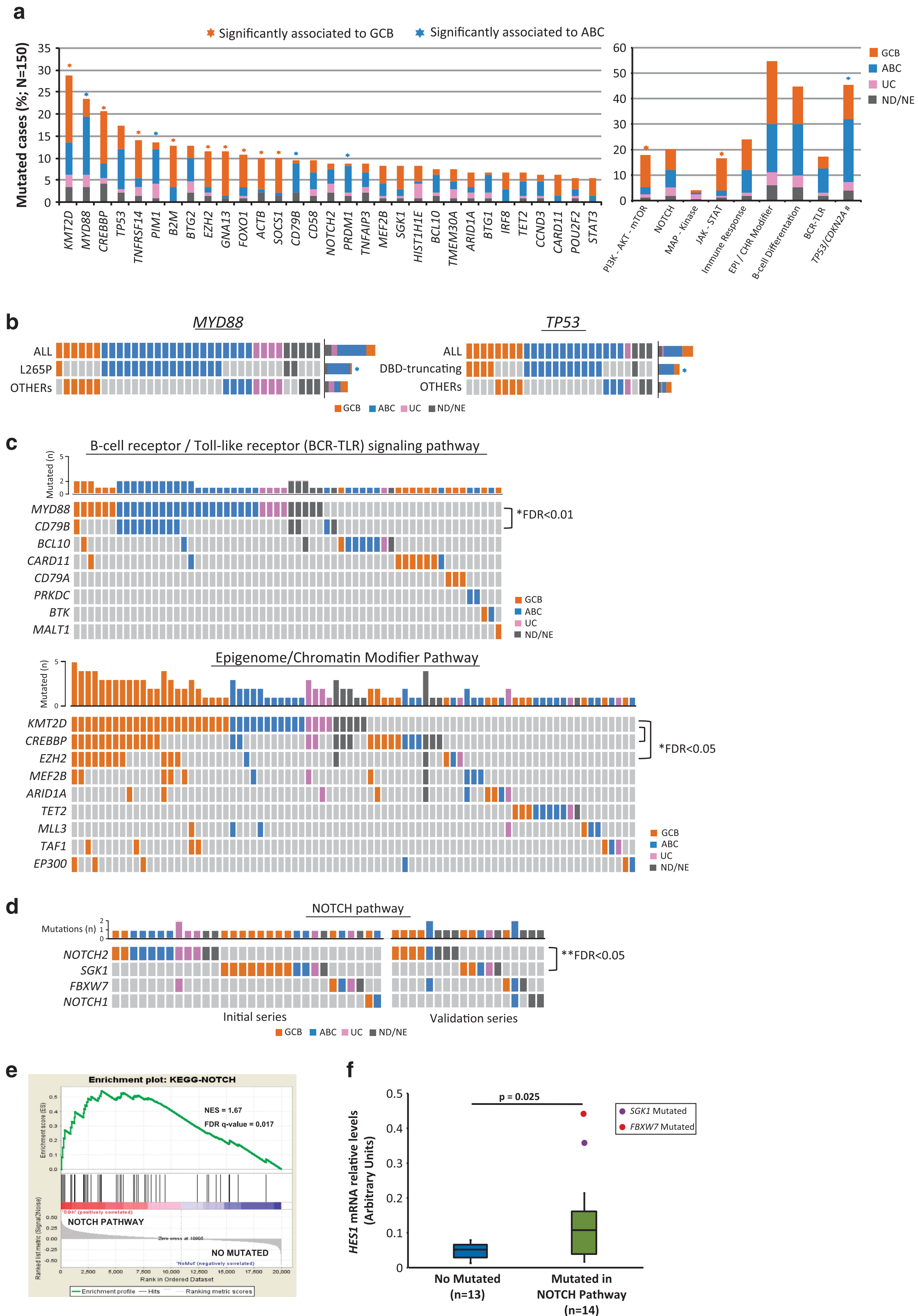
Clinical impact

The clinical impact of mutations and CNAs present in at least five cases was evaluated. Gains of 5p15, 11q24, 12q14 and 12q15 and losses of 8q12 correlated with lower CR rate, whereas no other CNA or single-gene mutation was associated with the response to therapy. As expected, R-IPI and COO among other standard clinical variables significantly predicted PFS and OS (Supplementary Table 9). Several mutated genes and CNA also had an impact on PFS and OS (Figure 3). Interestingly, among *TP53* variants, only truncating and DBD mutations were associated with shorter OS. Of note, *KLHL6* and *SGK1* were the only mutations associated with a worse OS independently of the IPI and COO of the tumor (Figure 3).

We then analyzed the clinical influence of genetic alterations in 10 predefined functional pathways or group of genes (Supplementary Table 6, Figure 3). The main features of the patients according to the aberrations in these pathways are listed in Table 2 and Supplementary Tables 10a and b. Alterations in NOTCH pathway (*NOTCH2*, *NOTCH1*, *FBXW7* and *SGK1*) and in *TP53/CDKN2A* were associated with shorter PFS and OS, whereas patients with *JAK/STAT* pathway (*SOCS1*, *STAT3 and STAT6*) mutations had superior OS (Figure 4a, Table 2). Alterations in

TP53/CDKN2A showed a trend for a worse response to therapy. A multivariate analysis including R-IPI (very good vs good vs poor) and COO (GCB vs ABC) along with NOTCH, TP53/CDKN2A and JAK/STAT pathways (non-altered vs altered in each case) showed in the final model with 82 cases that R-IPI (Hazard ratio (HR) 4.0; *P* = 0.006), NOTCH pathway (HR 2.8; *P* = 0.006) and TP53/CDKN2A (HR 2.4; *P* = 0.005) maintained independent significance for OS.

The prognostic impact of these three pathways was assessed in an independent cohort of patients. The distribution of the individual gene alterations was similar in both series



(Supplementary Table 11). The clinical features and outcome of the patients according to the status of NOTCH, TP53/CDKN2A and JAK/STAT pathways in the validation series are listed in Supplementary Table 12. As shown in Figure 4b, the adverse prognostic impact on OS of NOTCH pathway and TP53/CDKN2A alterations was validated in this independent cohort.

Genomic-guided therapeutic opportunities

We identified 69 (46%) cases carrying at least one genomic alteration in 9 genes (*CDK6, TP53, CDKN2A, PTEN, MYC, ARID1A* and *CD79B* (with or without *MYD88*), *EZH2* and *NOTCH1*) considered a biomarker of drug response as supported by the results of early clinical trials ($n=66$) or preclinical assays ($n=3$) in DLBCL or other

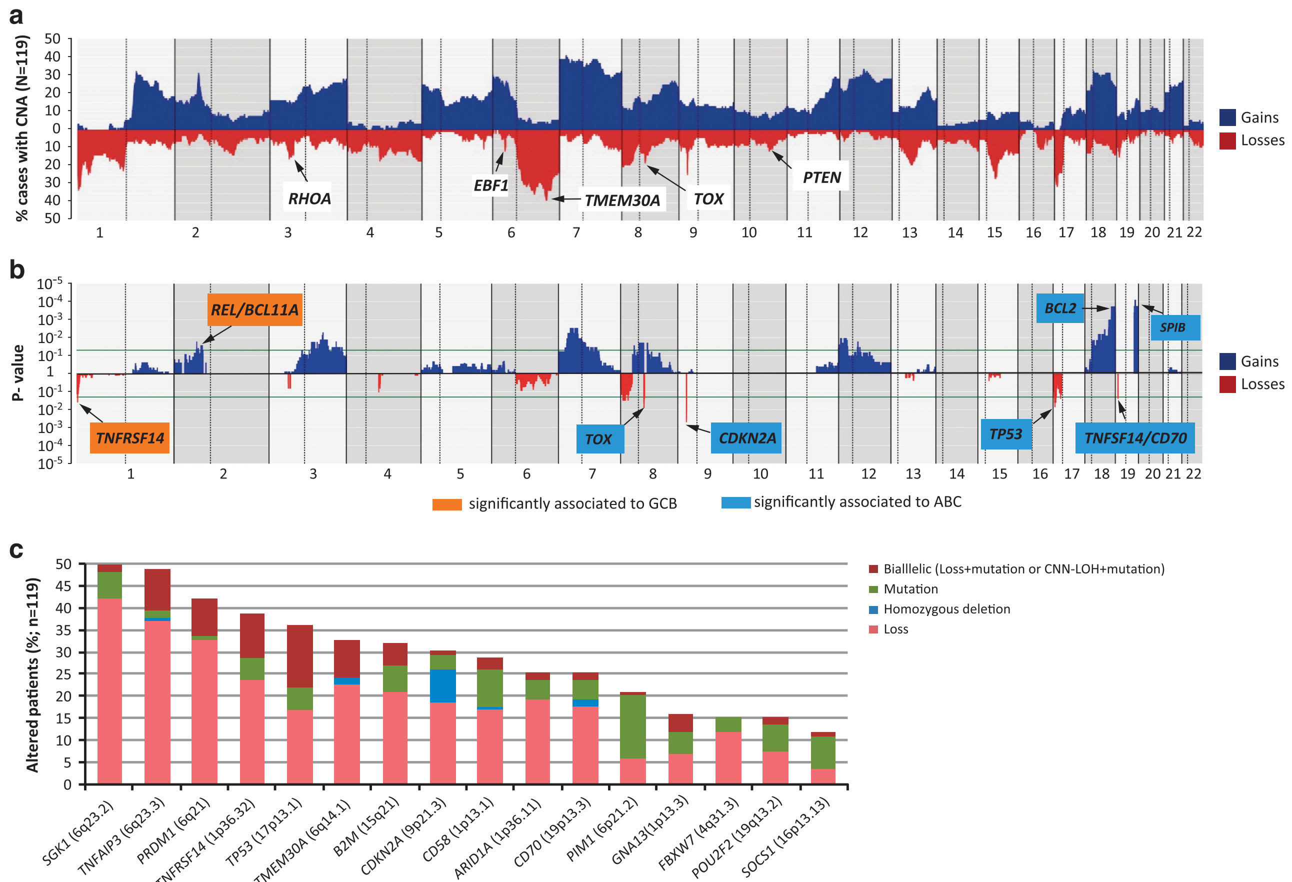


Figure 2. Copy number alterations (CNA) in 119 DLBCLs patients and integration with other genetic alterations. (**a**) Frequency of CNA of 119 DLBCL patients analyzed by Cytoscan HD assay. Each probe is aligned from chromosome 1 to 22 and p to q. Chromosomes X and Y were excluded from the analysis because sex-matched reference DNA samples were not used. The vertical axis indicates frequency of the genomic aberration among the analyzed cases. Gains are depicted in dark blue and losses are depicted in red. Genes affected by copy number alterations and not previously described in DLBCL are indicated. (**b**) Significant patterns of CNAs between DLBCL subtypes are depicted: ABC (light blue boxes) and GCB (orange boxes). The *X*-axis shows *P*-value among these two groups and significant threshold is marked with a green line. (**c**) Bar-graph represents frequency of mutations and CNAs for each gene in 119 DLBCL cases, determined by targeted NGS (*CDKN2A* by Sanger sequencing) and copy number analysis. Gene alterations are divided into four groups: Mutations (single-nucleotide mutations and/or small indels), homozygous deletion, loss (loss of one allele) and bialleic inactivation (Loss+mutation or CNN-LOH+mutation).

Figure 1. Recurrent mutated genes and pathways in 150 DLBCLs patients. (**a**) Bar-graphs show mutated genes in more than 5% of DLBCL patients and frequently mutated pathways. Each color bar indicates biological subtypes; GCB: germinal center B-cell type, ABC: activated B-cell type, UC: unclassified, ND/NE: not done or not evaluable. An asterisk represents mutated genes/pathways significantly enriched in one of the subtypes of COO and asterisk color denotes the enriched group. Tumor suppressor genes include mutations and deletions in *TP53* and *CDKN2A*, respectively. (#) (**b**) Heat maps show the distribution of *MYD88* and *TP53* mutated patients in both DLBCL subtypes. *TP53* mutations are divided into truncating and missense mutations located on the DNA binding domains (DBD) and 'others'. Columns depict individual cases and rows mutated genes/mutation type. (**c**, **d**) Heat maps representing relationships among mutated genes in B-cell receptor (BCR)/Toll-like receptor signaling, Epigenome/Chromatin Modifier and NOTCH pathways. Graph-bars above show the total number of mutated cases for each gene. One black asterisk represents significant mutated gene concurrence and two asterisks significant exclusion. Significant *P*-values corrected by false discovery rate (FDR) are showed. (**e**) Gene-set enrichment analysis (GSEA) of NOTCH pathway mutated cases vs cases with no mutations in genes of this pathway. (**f**) Box plots show *HES1* mRNA expression levels in NOTCH pathway mutated cases and cases with no mutations in this pathway.

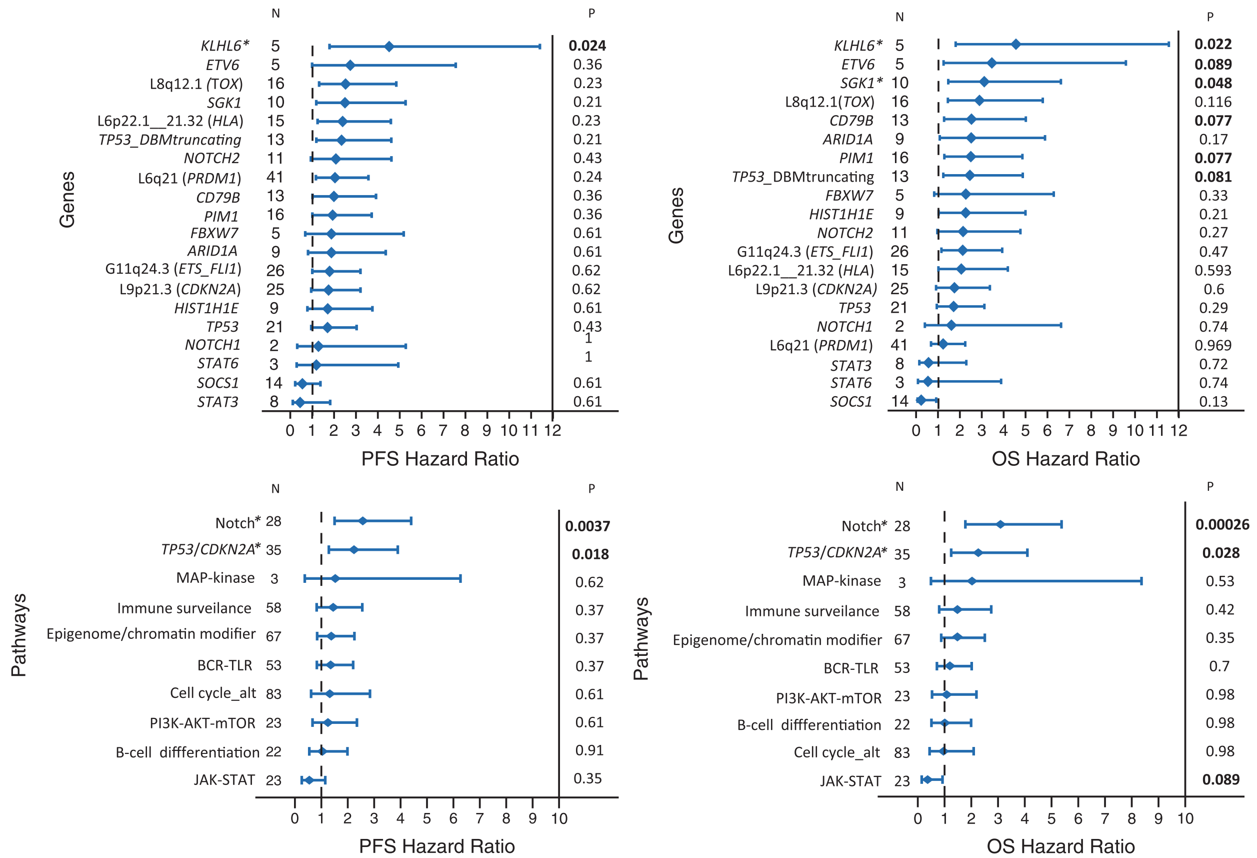


Figure 3. Forest plots of OS and PFS of gene alterations and pathways in the initial series. Gene alterations herein shown correspond to those with significant impact on overall (OS) or progression-free survival (PFS) in the statistical analysis before correction for multiple comparisons, as well as those drivers included in any of the three significant pathways. The *P*-values shown were corrected for multiple comparisons (Benjamini–Hochberg method). *indicates gene and pathway mutations that had prognostic value independent of the IPI and COO of the tumor in the multivariate analysis.

Table 2. Baseline features of the patients according to NOTCH, TP53/CDKN2A and JAK/STAT pathways in the initial series

Parameter	*NOTCH*		*TP53/CDKN2A*		*JAK-STAT*	
	Unmutated (N = 121)	*Mutated (N = 29)*	*Unmutated (N = 79)*	*Mutated (N = 43)*	*Unmutated (N = 125)*	*Mutated (N = 25)*
Age > 60 years	70/120 (60%)	21/29 (72%)	49/79 (62%)	28/42 (67%)	**85/124 (69%)**	**8/25 (32%)***
Male gender	63/121 (52%)	15/29 (52%)	43/79 (54%)	24/43 (56%)	64/125 (51%)	14/25 (56%)
Stage III/IV	59/119 (50%)	19/29 (66%)	49/79 (62%)	20/42 (48%)	66/123 (54%)	12/25 (48%)
ECOG 2 or higher	46/116 (40%)	10/29 (34%)	35/78 (45%)	16/41 (39%)	48/120 (40%)	8/25 (32%)
Extranodal involvement	58/119 (49%)	15/29 (52%)	37/79 (47%)	23/42 (55%)	62/123 (50%)	11/25 (44%)
Bone marrow involvement	11/118 (9%)	3/29 (10%)	4/79 (5%)	7/43 (16%)	13/125 (10%)	1/25 (4%)
High serum LDH	47/112 (42%)	16/29 (55%)	36/77 (47%)	19/39 (49%)	55/118 (47%)	8/23 (35%)
High serum B2m	53/99 (54%)	18/26 (69%)	41/71 (58%)	22/33 (67%)	**65/107 (61%)**	**6/18 (33%)***
HCV+	6/86 (7%)	4/24 (17%)	8/92 (9%)	2/18 (11%)	7/61 (11%)	2/29 (7%)
R-IPI score						
Very good	17/119 (14%)	1/29 (3%)	9/79 (11%)	4/42 (10%)	12/123 (10%)	6/25 (24%)
Good	56/119 (47%)	14/29 (48%)	32/79 (41%)	20/42 (47%)	58/123 (47%)	12/25 (48%)
Poor	46/119 (39%)	14/29 (48%)	38/79 (48%)	18/42 (43%)	53/123 (43%)	7/25 (28%)
COO						
GCB	49/100 (49%)	11/22 (50%)	**36/64 (56%)**	**9/37 (24%)***	**41/100 (41%)**	**19/22 (86%)***
ABC	46/100 (46%)	9/22 (41%)	**24/64 (38%)**	**26/37 (70%)***	**52/100 (52%)**	**3/22 (14%)***
UC	5/100 (5%)	2/22 (9%)	**4/64 (6%)**	**2/37 (6%)***	**7/100 (7%)**	–
CR rate[a]	78/101 (77%)	14/25 (56%)	53/66 (80%)	22/35 (63%)	74/103 (72%)	18/23 (78%)
5-year PFS[a]	**61%**	**23%****	**61%**	**34%***	52%	65%
5-year OS[a]	**68%**	**27%****	**68%**	**46%***	**57%**	**78%***

Abbreviations: ABC, activated B-cell; COO, cell of origin; CR, complete response; GCB, germinal center B-cell; OS, overall survival; PFS, progression-free survival; UC, unclassified. *$P < 0.05$; **$P < 0.01$. Entries in bold mean the differences are statistically significant. [a]Only patients treated with R-CHOP.

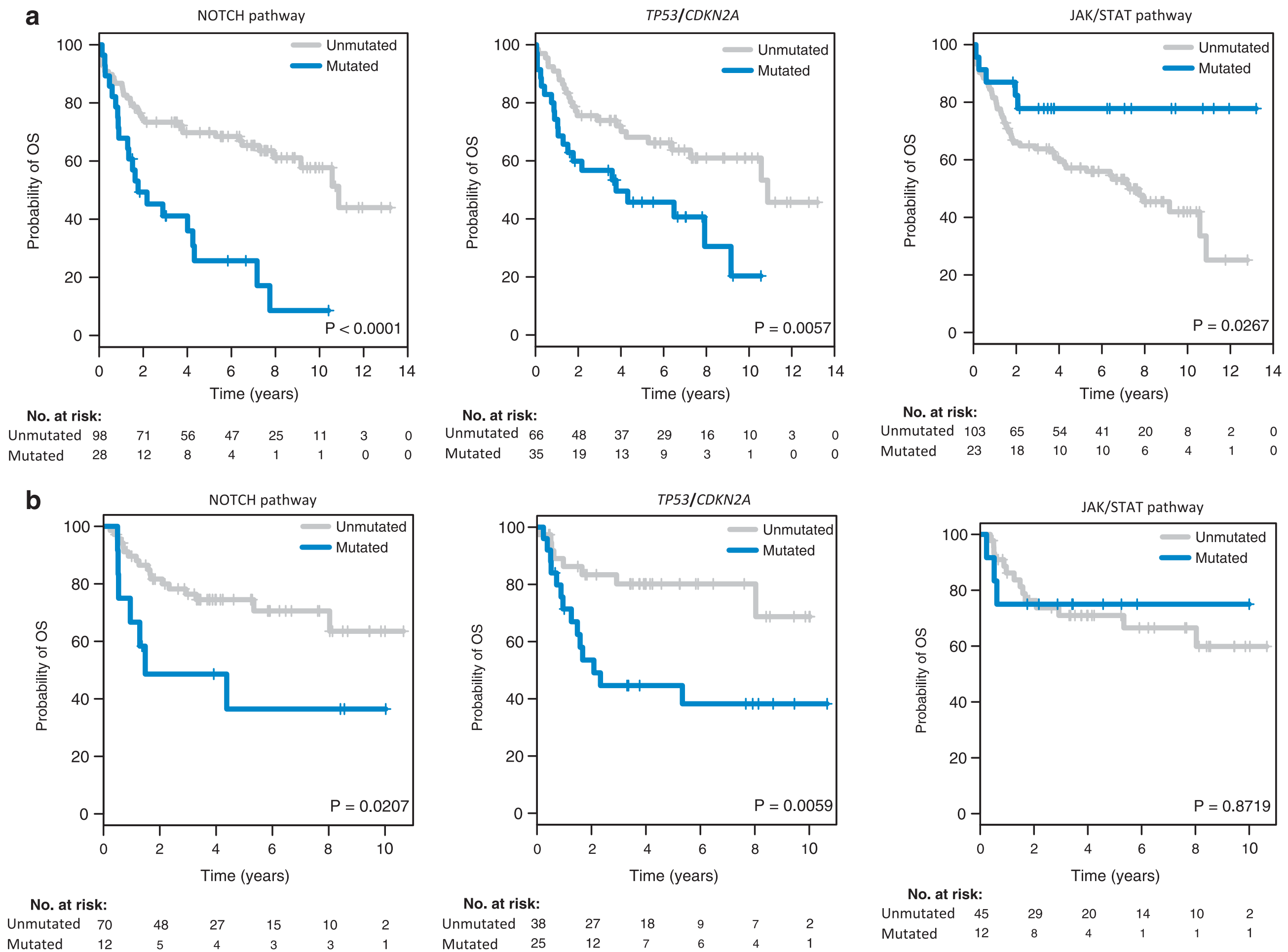


Figure 4. PFS and OS according to alterations in NOTCH and JAK-STAT pathways and TP53/CDKN2A (4A and 4B are for the initial and the validation series, respectively).

lymphomas (Supplementary Table 13) (Figures 5a–c). The tumors of 26 additional patients (17%) showed at least one gene alteration that could be exploited by a drug repurposing strategy of two types (Figure 5a). The first one corresponds to gene alterations that are biomarkers of drug response described in other cancer types and whose effect in DLBCL has not been assessed yet. The second one were mutations observed in genes described as biomarkers of drug response mostly in preclinical assays, but had different amino acid changes in the same functional domains. These mutations are predicted to have the same oncogenic effect as the known biomarker and may therefore lead to a similar drug response.

DISCUSSION

This study confirms the differential distribution of mutated genes, pathogenic pathways and CNA in GCB and ABC-DLBCL and also the presence of common alterations in both subtypes highlighting the molecular heterogeneity of these tumors. The larger number of cases investigated compared with previous whole-exome sequencing studies has expanded the view of the interactions among the individual mutated genes and those integrated in specific pathways. In this sense, we confirmed the association of *MYD88* mutations, particularly L265P, with *CD79B* mutations in ABC-DLBCL whereas other *MYD88* mutations occurred indistinctively in both DLBCL subtypes.[24,29] Similarly to other studies, *CREBBP* and *KMT2D* were found in both DLBCL subtypes.[10–13,30] However, here they were also significantly associated between them and with *EZH2* mutations in GCB-DLBCL, whereas *KMT2D* mutations, independent of the other two genes, were also detected in ABC-DLBCL. CREBBP and EP300 have a similar function and molecular structure.[31] Mutations in these genes have been found as mutually exclusive in DLBCL associated with adverse clinical outcome.[32] However, in our series most *EP300* mutations occurred in tumors with *CREBBP* mutations. None of these mutated genes had prognostic significance (Figures 1c and 3).

In addition to individual genes, we integrated the analysis of the mutations in different components of pathogenic pathways. Mutations in genes of the PI3K-AKT-mTOR pathway were significantly more frequent in GCB-DLBCL. This finding is consistent with the activation of PI3K signaling pathway observed in these tumors frequently associated with loss of *PTEN*.[7,33] JAK-STAT signaling is a feature of ABC-DLBCL triggered by autocrine production of Interleukin-6 (IL-6) and Interleukin-10 (IL-10).[24,34–36] However, we found mutations in *SOCS1*, *STAT3* and *STAT6* more frequently in GCB-DLBCL, a finding that expands the previous observation of inactivating *SOCS1* mutations in GCB-DLBCL.[37]

We also found frequent mutations in genes of the NOTCH pathway, *NOTCH2* (9%), and less frequently in *NOTCH1* (3%), that were confirmed in the validation cohort. All *NOTCH1* and *NOTCH2* mutations truncated the PEST domain. Mutations in these genes have only occasionally been detected in previous DLBCL whole-genome/exome sequencing studies, probably due to the relatively low coverage of these studies compared with ours (≈50× vs

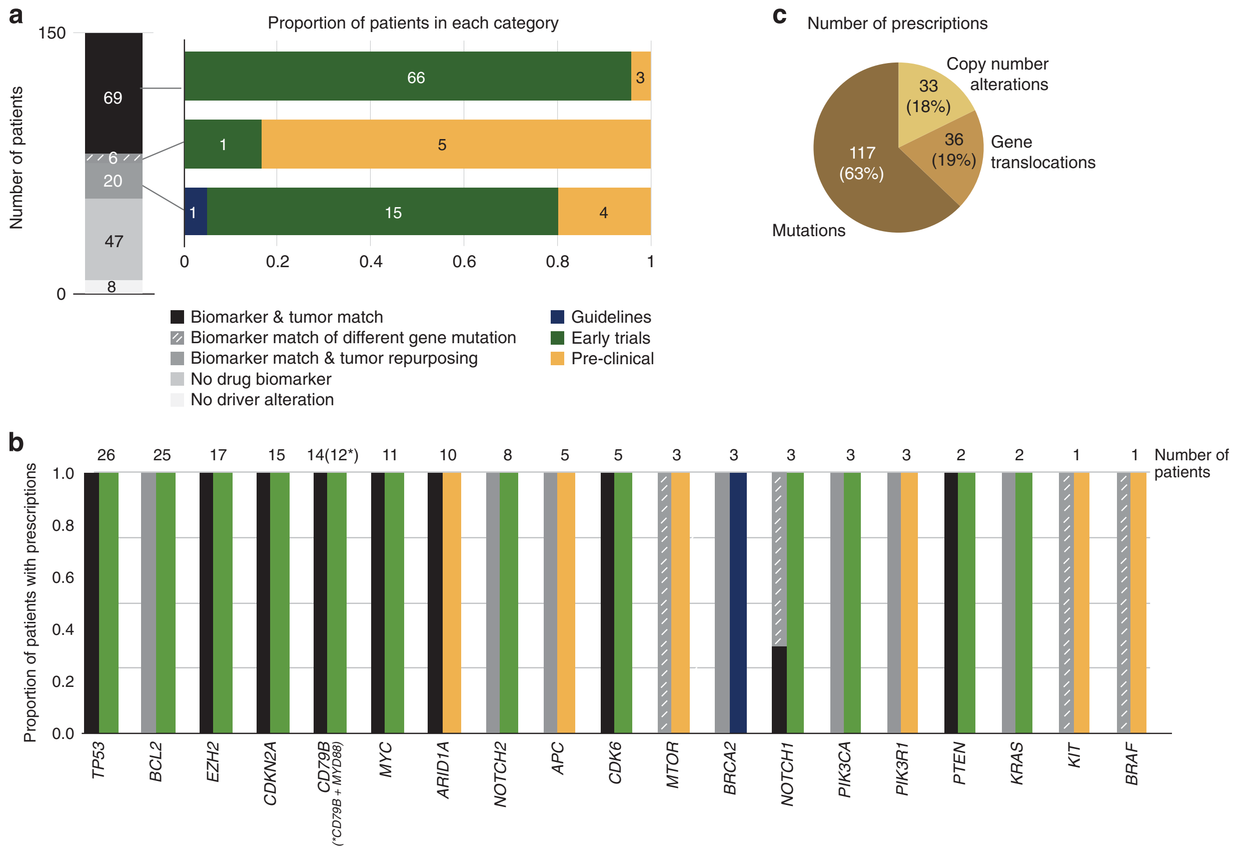


Figure 5. Genomic-guided therapeutic opportunities of the DLBCL cohort. Therapeutic opportunities have been classified according to the level of evidence supporting the effect of the genomic biomarker into (**i**) clinical guidelines (for example, FDA-approved or NCNN recommendations), (**ii**) late (phases III–IV) or (**iii**) early (phases I–II) clinical trials, (**iv**) case reports or (**v**) preclinical data. In addition to the alterations described as biomarkers of drug response in DLBCL (biomarker and tumor match), we included driver mutations in genes described as biomarkers of drug response in DLBCL upon a different amino acid change (biomarker match of different gene mutation), as well as genomic alterations described as biomarkers of drug response in other tumor types (biomarker match and tumor repurposing). (**a**) This panel depicts the therapeutic opportunities per patient (each patient has been counted only once according to their best therapeutic option following the above classification). (**b**) This panel depicts the therapeutic opportunities per gene; the numbers on top of the bars correspond to the number of patients exhibiting a biomarker of drug response in that gene (each patient has been counted only once according to their best therapeutic option given the gene alteration). Biomarkers that have been described for DLBCL and other non-Hodgkin lymphomas were also considered in the tumor match category. (**c**) Finally, this panel depicts the contribution of each alteration type to the overall number of *in silico* prescriptions per patient and altered gene.

$>500\times$, respectively,) and which may have been insufficient to detect mutations in the GC-rich hot-spot region.[10–13,38] We also sequenced the 3′UTR region of *NOTCH1* recently described as a hot spot for activating somatic mutations in chronic lymphocytic leukemia (CLL).[39] Only one case showed this type of mutation. We also found mutations in other genes of NOTCH pathway including the ubiquitin ligase *FBXW7* and the kinase *SGK1*.[25,26,40] To validate the role of this pathway in DLBCL, we investigated the expression of downstream genes regulated by NOTCH and observed that tumors carrying mutations in this pathway had a significant overexpression of NOTCH target genes. These findings support the role of the NOTCH pathway in a subset of DLBCL. Further studies are warranted to explore the impact of these mutations on DLBCL.

The integration of mutations and CNA has revealed new genes targeted by both types of alterations in DLBCL. In addition to biallelic alterations of known genes such as *CDKN2A*, *TNFAIP3*, *PRDM1*, *PTEN*, *B2M*, *CD70* or *CD58*, we also found novel biallelic alterations of *TMEM30A*, *SGK1*, *GNA13* and *EBF1*, among others, indicating the relevance of their inactivation in DLBCL. The frequent alterations of *TMEM30A* and *SGK1* identify these genes as new targets of the complex 6q14–q23 deletion in DLBCL, in addition to the known *PRDM1* and *TNFAIP3*. The role of *TMEM30A* in lymphomagenesis is unknown but it is interesting that its truncating mutations interfere with the function of ATP11C, a transmembrane protein involved in B-cell differentiation and BCR signaling[41,42] whereas *SGK1* inactivation seems to promote NOTCH signaling.[25,26]

Several studies have identified the prognostic value of individual mutated genes and CNA in DLBCL.[7,14,43–48] However, their significance is still controversial as very few of these alterations have been validated in independent cohorts. We confirmed the poor prognosis of *CDKN2A* deletions and 8p23.1 losses[49–52] in DLBCL. However, we could not validate the adverse effect of 3p gains, including *FOXP1*.[7] The clinicopathological significance of CNA, especially other than *CDKN2A*, should be further analyzed in DLBCL.

Our study identified the prognostic impact of several individual mutated genes after correction for multiple comparisons, but only *SGK1* and *KLHL6* were independent of R-IPI and the COO of the tumors. The integrated analysis of alterations in pathogenic pathways has an increasing interest for strategies targeting mechanisms rather than individual genes.[53,54] This approach may also overcome the challenges of the low frequency of most mutated genes in DLBCL.[53,55] In this perspective, we found that genetic alterations in NOTCH pathway and *TP53/CDKN2A* genes conferred poor outcome

independently of the R-IPI and COO, and these findings were confirmed in the independent cohort of DLBCL.

Recent studies have revealed the relationship between *NOTCH1/2* mutations and tumor aggressiveness in different mature B-cell neoplasms including CLL,[39,56] splenic marginal zone lymphomas,[57,58] follicular lymphomas[59,60] and mantle cell lymphomas.[19,61] In our DLBCL cohort none of the individual mutated genes of the pathway had a confirmed prognostic significance in the initial and validation cohorts. On the contrary, the integrated analysis of the mutations in all the genes of the pathway conferred an adverse prognosis that was independent of the IPI and COO subtype. This finding was confirmed in the validation cohort supporting the relevance of NOTCH pathway in DLBCL and indicating that the integrated analysis of altered pathways in DLBCL may be more relevant than individual genes.

We observed a similar situation with *TP53* and *CDKN2A* alterations. As previously observed,[62] not all *TP53* mutations had a prognostic impact. In the initial cohort only *TP53* truncating and DBD mutations were associated with a significant shorter survival, although it was not independent of the COO subtype. *TP53* mutations occurred in both ABC and GCB subtypes but truncating and mutations in DBD occurred preferentially in ABC-DLBCL. Interestingly, the combination of *TP53* truncating and DBD mutations, and *CDKN2A* deletions was associated with adverse prognosis that was independent of the R-IPI and COO of the tumor and could be confirmed in the independent validation cohort.

We also identified a subset of tumors with mutations in JAK/STAT pathway that had a better outcome. Activation of the JAK-STAT is common in primary mediastinal large B-cell lymphoma, but we specifically excluded these tumors in our study. Interestingly, the good prognosis of DLBCL with *SOCS1* inactivating mutations has been previously observed.[14,32,37] Although the prognostic value could not be validated in the independent cohort, identifying these patients as candidates for targeted therapies may be relevant.[54]

The comprehensive profiling of genomic alterations in this DLBCL cohort revealed a landscape of genomic-guided therapeutic opportunities.[54] Overall, 46% of the tumors exhibited biomarkers of drug response currently supported by the results of early clinical trials (phases I/II) or preclinical assays. This number is extended to 63% when drug repurposing opportunities are also taken into account. This analysis concentrated on drugs interacting directly with altered genes. Further studies considering drugs with potential effect on pathogenic altered pathways may expand the number of patients who could benefit from a personalized approach. The consideration of these therapeutic strategies may open new perspectives for patients suffering from tumors unresponsive to standard strategies.

In conclusion, we have recognized novel target genes and defined the relevance of alterations of NOTCH pathway and *TP53/CDKN2A* in DLBCL. Our findings suggest that the global analysis of alterations in defined pathways may be more relevant than independent genes. Using an *in silico* prescription pipeline we have also identified a number of candidate drugs with potential therapeutic interactions with driver oncogenic proteins. All these findings may orient future preclinical and clinical intervention strategies in DLBCL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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