Genetic Landscapes of Relapsed and Refractory Diffuse Large B-Cell Lymphomas


Abstract

**Purpose:** Relapsed or refractory diffuse large B-cell lymphoma (rDLBLCL) is fatal in 90% of patients, and yet little is known about its biology.

**Experimental Design:** Using exome sequencing, we characterized the mutation profiles of 38 rDLBLCL biopsies obtained at the time of progression after immunochemotherapy. To identify genes that may be associated with relapse, we compared the mutation frequency in samples obtained at relapse to an unrelated cohort of 138 diagnostic DLBLCLs and separately amplified specific mutations in their matched diagnostic samples to identify clonal expansions.

**Results:** On the basis of a higher frequency at relapse and evidence for clonal selection, TP53, FOXO1, MLL3 (KMT2C), CCND3, NFKBIE, and NFKBIZ, and STAT6 emerged as top candidate genes implicated in therapeutic resistance. We observed individual examples of clonal expansions affecting genes whose mutations had not been previously associated with DLBLCL including two regulators of NF-kB: NFKBIE and NFKBIZ. We detected mutations that may affect sensitivity to novel therapeutics, such as MYD88 and CD79B mutations, in 31% and 23% of patients with activated B-cell type of rDLBLCL, respectively. We also identified recurrent STAT6 mutations affecting D419 in 36% of patients with the germinal center B (GCB) cell rDLBLCL. These were associated with activated JAK/STAT signaling, increased phospho-STAT6 protein expression and increased expression of STAT6 target genes.

**Conclusions:** This work improves our understanding of therapeutic resistance in rDLBLCL and has identified novel therapeutic opportunities especially for the high-risk patients with GCB-type rDLBLCL.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin lymphoma (NHL) and is curable in approximately 60% of patients with multi-agent chemotherapy, such as rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP; ref. 1). DLBLCL can be classified into two cell-of-origin (COO) molecular subtypes, the germinal center B (GCB) type and activated B-cell (ABC) type, where the latter is associated with an inferior outcome (2, 3). DLBLCL can also evolve from histologic transformation of a pre-existing indolent lymphoma (TLy), most commonly follicular lymphoma. Patients with relapsed or refractory TLy (rrTLy) and DLBLCL (rrDLBCL) have a poor outcome, with only approximately 40% of patients achieving long-term remissions with salvage chemotherapy and autologous stem cell transplant (SCT; ref. 4).
The genetic landscape of de novo DLBCL is well characterized. Genomic alterations contribute to escape from immune surveillance, inhibit DNA damage response and apoptosis, promote survival pathways, or reprogram the epigenetic landscape of the tumor (5–16). Although DLBCL shares some commonalities across COO types, the mutation frequency of some genes is enriched only in one subtype. Genomic analysis of paired follicular lymphoma and TLY samples revealed that TLY is genetically more complex than follicular lymphoma and has genetic features associated with both GCB-DLBCL and ABC-DLBCL (17, 18). However, most TLY samples analyzed to date were collected after chemotherapy, making it challenging to differentiate whether genetic changes distinguishing de novo GCB-DLBCL from TLY contribute to histologic transformation or result from clonal selection induced by chemotherapy (17, 18).

Subsequent to a selective pressure, such as R-CHOP treatment, chemotherapy-resistant subclones that survive may be enriched at the time of relapse due to clonal expansions. Obtaining a biopsy at the time of progression provides an opportunity to identify genomic alterations unique to these populations of cells. However, rDLBCL samples are rare because patients are not routinely biopsied at the time of relapse outside the context of a clinical trial. Given that very few patients survive rDLBCL, there is also a lack of constitutional DNA for most archived samples, which is important to exclude germline variants when performing whole-exome sequencing (WES). For such reasons, the genomic profile of rDLBCL is not well known, with two small cohorts of approximately 10 patients described in the literature to date (19, 20). Characterizing a sufficient number of rDLBCL samples may lead to insights into the genetic events that lead to therapeutic resistance and reveal pathways that are consistently activated at relapse, some of which may be targeted with existing or novel therapies.

Herein, we report the mutational landscape of 38 samples of rDLBCL and rTLY, determined using WES. Many genes mutated in this cohort have been reported with variable frequencies in diagnostic DLBCL. TLY, primary mediastinal B-cell lymphoma (PMBCL), and follicular lymphoma (5, 7–10, 17, 18, 21, 22). On the basis of comparison with published data on de novo DLBCL and evidence for clonal expansion in paired biopsies, we find a higher prevalence of mutations affecting six genes at relapse, including the transcription factor STAT6. The recurrent mutation we observe affecting the D419 residue has been reported to activate JAK/STAT signaling in follicular lymphoma and conferred sensitivity to JAK2 inhibitors in PMBCL (22, 23). We show that STAT6 D419 mutations may similarly activate JAK/STAT signaling in the GCB-rDLBCL and rTLY (22). With the exception of PMBCLs, this pathway has not previously been implicated in de novo DLBCL.
Immunohistochemical analysis

For 25 of 27 of the clinical trial patients, slides containing 5 µm of FFPEt were used to determine the protein expression of phospho-STAT6 (rabbit polyclonal anti-Tyr641; Cell Signaling Technology) using an automated Ventana system (Roche) and >10% of cells was considered positive using previously published thresholds (23). Tumor content was estimated by the number of tumor cells by hematoxylin and eosin stain and CD20 expression (clone L26; Dako) in the QCROC-2 cases prior to B-cell enrichment (Supplementary Table S1). Because of the low quantity of cells obtained after B-cell selection, tumor content was not reassessed. In patients where gene expression profiling was not available or called “unclassifiable,” COO assignment was determined on the basis of Hans criteria (26).

Exome sequencing and analysis

Tumor and germline samples were sequenced using exome capture in 38 patients with 100 nt paired reads using the HiSeq 2000 platform (Illumina). Somatic variants were detected using Strelka with the depth filter disabled (27). We implemented a custom filter to discount variant base calls deriving from the same fragment to mitigate an increased rate of false variant calls (27). We also implemented a custom filter to discount variant base calls deriving from the same fragment to mitigate an increased rate of false variant calls (27). The average mutation load was higher in the trial samples relative to the archival rrDLBCLs (119.1 ± 7.26 × 10−3) × 106 vs. (115.7 ± 12.81 × 10−3) × 106). The most common mutations were evenly distributed between the clinical and archival rrDLBCL cohort combining clinical trial and archival rrDLBCLs.

Targeted sequencing of candidate genes

We performed targeted sequencing in 30 of 38 rrDLBCL samples to determine the variant allele fraction (VAF) of genes that were frequently mutated in the exomes and/or had a role in diagnostic DLBCL (provided in Supplementary Methods). FOXO1 was included because it is known to be associated with a poor outcome in DLBCL and had poor coverage across most exomes (29). In 12 patients, we performed deep sequencing of selected mutations in paired DLBCL biopsies (diagnosis/relapse) taken from the same patient. Targeted sequencing of STAT6 and other genes was also performed in the “pretreatment” cohort of 49 patients to determine the frequency of these mutations obtained using a deep-sequencing approach in diagnostic DLBCL and rrTly samples obtained prior to chemotherapy.

Gene expression profiling and gene-set enrichment analysis

The isolated RNA from purified lymphoma cells that was used in cDNA microarray analyses using either the Sureprint 8 × 60K one-color human expression array (Agilent) or the Human Gene 2.0 ST array (Affymetrix) platforms. We calculated the COO (L1MP score) using the subgroup of predictor genes identified by Wright and colleagues (30). Refer to Supplementary Methods for the methodology of normalization of platforms and the gene expression analysis of STAT6-mutant rrDLBCL.

Results

The mutational spectrum of relapsed or refractory DLBCL and Tly

The mutational profiles of the 38 rrDLBCL and Tly samples illustrated in Fig. 1 demonstrates that genetic heterogeneity is a hallmark of this disease, even following treatment. The complete exome and targeted sequencing data are found in Supplementary Tables S3 and S4, respectively. The average mutation load was higher in the trial samples relative to the archival rrDLBCLs (119.1 ± 63.7 non silent SNVs, respectively; P = 0.032), but the most common mutations were evenly distributed between both groups, which were combined for subsequent analyses. We also identified somatic copy number alterations (CNA) using the WES data and observed many events known to be common in de novo DLBCL (Supplementary Fig. S1).

The genes commonly mutated at relapse and additional ones known to contribute to the pathogenesis of de novo DLBCL were ranked in decreasing order of frequency in Table 1. The genes most commonly mutated in ABC rrDLBCL were FOXO1 (38%), MLL3 (31%), MLL2 (23%), and CD79B (23%). The most common GCB rrDLBCL–enriched mutations were CREBBP (45%), STAT6 (36%), and FOXO1 (36%) followed by BCL2 and EZH2 in 27% of samples. The most common mutations that were not COO-specific were TP53 (32%), MLL2 (KMT2D) (28%) and FAS (16%). Given their known roles as tumor suppressor genes, FAS, MLL2, TP53, and CREBBP bore a general pattern of inactivating mutations. In contrast, MYD88 (L265), EZH2 (Y641), FOXO1, and STAT6 were genes in which mutations recurred at hot spots, indicating they were likely functionally significant (6, 15, 21, 29).

The mutation profile of rrTly was very similar to GCB-rrDLBCL. Tly represented mainly transformed follicular lymphoma (11/13 cases), all of which had a GCB phenotype. One case arose from a marginal zone lymphoma and classified as GCB. The last case arose from Waldenstrom macroglobulinemia, which harbored a MYD88 mutation and was classified as ABC. Mutations in STAT6 and FAS, two genes that were previously reported to be a consequence of histologic transformation of follicular lymphoma, were equally distributed in GCB-type rrDLBCL and rrTly (17, 18). In contrast, there was a higher frequency of FOXO1 mutations in GCB-rrDLBCL relative to rrTly cases. Thus, even in the relapse setting, the genetic profile of ABC-rrDLBCL is different from GCB-rrDLBCL and rrTly.

Clonal selection for driver mutations

To search for genes with a potential role in therapeutic resistance, we used two complementary approaches. First, we compared the mutation frequency obtained by WES in the “relapse” rrDLBCL cohort (n = 25) to a separate “diagnostic” rrDLBCL cohort (n = 138) and excluded the 13 cases of rrTly for this analysis. On the basis of this comparison, seven genes were significantly more commonly mutated among the “relapse” samples, namely: MLL3, MIPG1, NFKBIZ, CCND3, STAT6, TP53, and MYC (P < 0.05; Table 1). The mutations in MYC and CCND3 affected hot spots previously reported in Burkitt lymphoma, where mutations have been shown to increase protein stability, such as I290R and Q276X in CCND3 and T73A and P74A in MYC (31, 32). Because of systematic low coverage of FOXO1 among exomes (Supplementary Figs. S2 and S3), we separately compared the prevalence of FOXO1 mutations in our rrDLBCLs to a large published cohort of 279 untreated DLBCLs (29). This revealed a significant
enrichment of FOXO1 mutations among rrDLBCLs (8.6% vs. 27%, \(P = 0.022\); ref. 29), thereby nominating this as an eighth gene for which mutations may be more common at relapse.

We then analyzed 12 tumor pairs derived from the same patients, where one sample was obtained at diagnosis and one at relapse and determined whether mutations found at relapse were acquired or resulted from expansion of subclones present at diagnosis. We calculated the VAF for selected mutations, which is proportional to the fraction of cells in the tumor carrying a given mutation, but can also be affected by tumor purity and CNV. Figure 2 illustrates that there are variations in VAF for different mutations at diagnosis and at relapse, suggesting that clonal evolution can occur following RCHOP therapy. We found evidence for clonal expansions of at least one mutation in 11 of 12 paired-samples affecting mutations in 23 genes (Table 2; Supplementary Fig. S4). Some mutations were present, but subclonal at diagnosis, and subsequently appeared in a larger fraction of cancer cells at relapse. This was observed for some of the mutations in BCL2, as well as mutations affecting RB1, EZH2, MYD88, NFKBIE, FOXO1, TNFRSF14, CARD11, B2M, and STAT6. We also found examples of mutations that were detectable and often clonal at relapse, but were undetectable in the diagnostic specimen. This was the case for additional BCL2, STAT6, FOXO1, and TNFRSF14 mutations along with those affecting TP53, CD79B, NFKBIZ, SOCS1, MLL3, MEF2B, ARID1A, CCND3, MLL2, and TBL1XR1.

Taken together, our data support that clonal expansion of some mutations can occur following RCHOP. Importantly, many of the mutations present in the dominant clone at relapse could not be detected by deep sequencing the diagnostic biopsies of these same patients, indicating that these either existed in an exceedingly small subpopulation of cells, a separate population of cells not sampled by the biopsy, or that they were acquired de novo in the intervening time between diagnosis and relapse. Overall, we found multiple examples of tumors exhibiting clonal expansion of STAT6 mutations (3 cases) or other genes in this pathway (e.g., SOCS1, 2 cases), as well as those affecting FOXO1 (3 cases), NFKBIE (2 cases), MLL2 (2 cases), and CD79B (2 cases).

On the basis of prevalence at relapse and examples of clonal expansions, mutations in multiple genes emerged as potential candidates for contributing to therapeutic resistance in DLBCL. By considering enrichment in our rrDLBCL cohort in conjunction with clonal selection, we became particularly interested in TP53, FOXO1, MLL3, CCND3, NFKBIZ, and STAT6. Notably, in some cases, there was also evidence of copy number variation at relapse.
but our methodology was not capable of ascertaining whether each CNV was present in the diagnostic specimen (Supplementary Fig. S5).

Novel mutations affecting NF-κB in rrDLBCL

Multiple genes with roles in NF-κB signaling showed either increased mutation prevalence in our cohort and/or evidence for clonal enrichment at relapse including CARD11, CD79B, MYD88, NFKBIE, and NFKBIZ. The latter two genes were mutated in 7/38 (18%) of rrDLBCLs representing both GCB and ABC cases. Despite the known role of NF-κB in ABC-type de novo DLBCLs, neither gene has previously been associated with de novo DLBCL. *NFKBIE* encodes IκBε, which inhibits NF-κB-mediated transcription (33). Loss of IκBε is known to increase B-cell proliferation via autocrine IL6 signaling (34) and inactivating mutations in *NFKBIE* have been reported in Hodgkin lymphoma (HL) and chronic lymphocytic leukemia (CLL; refs. 35–37). All three NFKBIE-mutated rrDLBCLs in our cohort carried the same frameshift indel, suggesting a strong selection for this variant to inactivate this gene (Supplementary Fig. S6). This is the same 4-nt deletion mutation that has recently been reported in aggressive cases of CLL (37). We sequenced NFKBIE in 27 additional cases of untreated DLBCLs and detected two additional examples of this indel and two other missense SNVs (Supplementary Table S5). NFKBIZ encodes IκBζ, which regulates a set of NF-κB target genes that have been shown to promote the survival of ABC-DLBCL (38). The pattern of mutations affecting NFKBIZ were not clearly inactivating, comprising missense mutations largely restricted to the Ankyrin repeats. Taken together, the observation of mutations in genes involved in regulating NF-κB, including two genes with no previously established role in DLBCL, warrants further exploration on the role of these genes in the context of relapse.

Selection for mutant STAT6 D419 in post-treatment TLY and GCB-DLBCL

We further examined the potential role of STAT6 given its novelty in the context of *de novo* DLBCL and its reported role in JAK/STAT signaling, thereby nominating it as a potential therapeutic target. Of the nine STAT6 mutations detected in our relapse cohort, all but one affected a single amino acid (D419) in the highly conserved site within the DNA-binding domain. D419 is a reported polymorphism in online databases, but in the current study by Trinh and colleagues (29).

### Table 1. Frequency of mutations in relapsed or refractory DLBCL and TLY

<table>
<thead>
<tr>
<th>Gene</th>
<th>% Mutations rrDLBCL N = 25</th>
<th>% Mutations ABC N = 13</th>
<th>% Mutations GCB N = 11</th>
<th>% Mutations TLY N = 13</th>
<th>% Mutations Dx DLBCL N = 138</th>
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NOTE: These genes were selected and ranked based on their overall frequency by exome sequencing in rrDLBCL that excluded cases of TLY.

Abbreviations: Dx, diagnosis; TLY, a DLBCL arising in the context of histologic transformation from an indolent lymphoma.

*a* Genes indicated in the table above are significant at a threshold of P < 0.05. Raw P values are as follows: MPEGP: P = 0.020; MLL3: P = 0.020; CCND2: P = 0.026; STAT6: P = 0.033; TP53: P = 0.033; NFKBIE: P = 0.025; and MYC: P = 0.047.

*b* The frequency of FOXO1 mutations is based on targeted sequencing of 18 QC2-rrDLBCL samples and the frequency in 279 diagnostic samples based on the study by Trinh and colleagues (29).

*c* In one rrDLBCL sample, the molecular subtype was not available, thus it was not included in the GCB or ABC categories. That case harbored mutations in MLL2, MLL3, and MYC.
of 27 (15%) of de novo DLBCLs and 4 of 22 (18%) TLYs, confirming these mutations are not restricted to TLY. We found evidence for intratumoral heterogeneity in a single TLY tumor that harbored two separate STAT6 mutations. The VAF for one of these (E382K) was sufficiently high to indicate clonal representation (22.5%), whereas the VAF for the second mutation (D419G) was 2%, suggesting the presence of a subclone bearing this mutation. Interestingly, the pattern of STAT6 mutations in untreated samples appeared different than that observed at relapse. Among the "relapse" tumors, 8 of 9 mutations affected D419, whereas in pretreatment samples, the mutation pattern was more heterogeneous, similar to that reported in PMBCL (Fig. 3A; ref. 21).

Somatic Mutations in Relapsed DLBCL

Figure 2.
Exploration of clonal expansions using matched diagnostic tumors. Deep amplicon sequencing of selected mutations in paired diagnostic and relapsed tumors across 12 patients (A–L) revealed multiple examples of clonal selection for known or suspected driver mutations including multiple examples of variants affecting JAK/STAT signaling. In QC2-34, there was no evidence for the mutant STAT6 allele at diagnosis, whereas the VAF for other mutations likely to be clonal at diagnosis ranged from 6% to 13%, likely owing to low tumor content in this specimen. The STAT6 mutation was therefore either subclonal at diagnosis and evaded detection, or was acquired in the intervening time prior to relapse. In QC2-04, it is evident that the reduced VAF in mutant STAT6 in the diagnostic specimen (5%) was not due to tumor cellularity by observing a substantially higher VAF for a second mutation in the same sample (CREBBP, 28%). CNV can affect VAF and, given that LOH was detected in CREBBP in the relapsed tumor, the increase in CREBBP-VAF in that sample could be explained by this event. The large change in VAF for STAT6, however, can only be explained by this existing as a subclonal mutation at diagnosis. A third patient (QC2-39) harbored two mutations in the STAT6-regulator SOCS1 in T2. One of these mutations was completely undetectable in the diagnostic tumor while a third mutation in this gene (absent at relapse) was found. Other examples of clonal expansions affected driver mutations associated with poor outcome in DLBCL or follicular lymphoma such as FOXO1, TNFRSF14 and TP53. For example, QC2-07 showed acquisition of mutations in each of TP53, GNA2 and FOXO1. Although the MLL2 mutation was preserved, none of these additional mutations were detectable in the diagnostic biopsy. In MT-260, mutations in both HIST1H2E and TNFRSF14 were acquired and became clonal at relapse. A third acquired mutation, affecting FOXO1, was also acquired. Interestingly, this patient's tumor harbored two clonal FOXO1 mutations at diagnosis. Counter-examples in which mutations affecting these genes were present at diagnosis but other diver mutations were acquired are also shown, including QC2-35 (NFKBIZ), QC2-20 (RB1) and QC2-25 (CD79B and MLL3). Also included are examples of patients in which evidence for clonal selection was unclear due to confounding issues of tumor purity (QC-32, QC2-18). For comparison, the VAFs that have been corrected for tumor content estimates and/or determined using a separate (capture-based) sequencing strategy are included in Supplementary Fig. S4 and Supplementary Table S6.

JAK/STAT signaling is a potential target in GCB rrDLBCL and TLY
To address the impact of STAT6 mutations in rrDLBCL, we assessed the presence of activated nuclear phosphorylated STAT6 protein (phospho-STAT6) using 25 of 27 rrDLBCL FFPEP samples. Phospho-STAT6 was observed in 6 of 25 cases (24%), which was nuclear in 5 of 6 cases, and cytoplasmic in the other (Fig. 3B). Of the five nuclear cases, four had mutations in STAT6, and one had three mutations in SOCS1, which encodes a negative regulator of STAT6. The case with cytoplasmic phospho-STAT6 was wild type for both STAT6 and SOCS1. Only one STAT6 mutation was detected in the 18 phospho-STAT6–negative cases. Hence, the presence of STAT6 mutations was significantly associated with the...
presence of nuclear phospho-STAT6 protein ($P = 0.021$), which is consistent with these mutations contributing to STAT6 activation.

To determine the transcriptional impact of mutations in this pathway, we compared the gene expression profile of patients with mutant and wild-type STAT6. We restricted the analysis to the GCB cases because STAT6 mutations were present only in GCB and TLy cases. Given its known role in regulating this pathway and the presence of phospho-STAT6 in this case, we also included the single SOCS1 mutant in the STAT6-mutant cohort. We first performed gene-set enrichment analysis on these groups using predefined gene sets for JAK/STAT signaling. JAK/STAT signaling was enriched in the STAT6/SOCS1–mutant samples compared with those lacking such mutations ($NES = -1.31$; $FDR = 0.147$). The heatmap (B3C) demonstrated increased JAK/STAT signaling among the mutant DLBCLs, supporting previous reports that STAT6 mutations activate STAT6 and potentiate JAK/STAT signaling (22). Specifically, the mRNA expression of FCER2, IL2RA, and SOCS1, known STAT6 target genes from the KEGG pathway were higher in mutants. Of the seven genes identified as specific mutant STAT6 targets by Yildiz and colleagues, we had data on three, of which two were increased, but only IL2RA was statistically significant (22). In total, 10 of 24 (40%) of the cases that were either TLy (5/13) or CCB-rDLBCL (5/11) had mutations in either STAT6 or SOCS1, both of which were associated with active phospho-STAT6 protein and increased JAK/STAT6 signaling. Taken together, this pathway appears to be activated in rDLBCLs bearing mutations in STAT6 or its regulators and therefore, may represent a new target for treatment in the relapse setting.

### Discussion

This study provides the genetic profile of the largest cohort of rDLBCL to date and to our knowledge, the first time that exome sequencing was performed in rDLBCL samples obtained within the context of a clinical trial. We show that known lymphoma-related genes, as well as some novel genes not previously associated with de novo DLBCL, were frequently mutated at the time of relapse and, in some cases, significantly more than in untreated DLBCL patients. In addition to being more commonly mutated in this cohort, each of TP53, MLL3, NFKBIZ, STAT6, FOXO1, and CCND3 were also found to have undergone clonal enrichment between diagnosis and relapse. Our combined exome and targeted sequencing approach also identified novel and subclonal mutations that may have otherwise gone undetected if sequencing

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**Table 2. Selected mutations showing clonal evolution between diagnostic (T1) and relapse (T2) tumors**

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**NOTE:** Suspected driver mutations identified in each tumor (T2) sample were selected for sequencing in T1 to assess their presence and clonal abundance at the time of diagnosis. Patients for which at least one mutation showed clear evidence for clonal evolution relative to other variants in the tumor are included. VAF changes likely resulting from variability in tumor cellularity between the samples are not shown.

Abbreviations: Chr, chromosome; T1, tumor at diagnosis; T2, tumor at relapse; VAF, variant allele fraction; AA, amino acid change where ‘—’ predicts for a truncated protein.

*Adjustment of VAFs for tumor purity estimates affects whether these mutations underwent clonal expansion (see Supplementary Fig. S4). After adjustment, the VAF for the EZH2 mutation in QC2-32 clearly increased at relapse, whereas the NFkBIE mutations in QC2-39 appear stable following correction for tumor purity.

*Additional mutations in this gene with similar VAF changes were detected in this sample and a representative is shown here.

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Somatic Mutations in Relapsed DLBCL

Figure 3.
Activation of the JAK/STAT pathway in STAT6 and SOCS5-mutant lymphoma cells. A, in all but one of the STAT6-mutated rDLBCL cases interrogated either by exome or targeted sequencing (n = 7/8), the mutation affected at a single amino acid (D419) in the DNA-binding domain (DBD). In these samples, this residue was replaced by any of histidine (1), alanine (1), glycine (2), and asparagine (2). Although mutations in this residue have been observed in PMBCL, the other mutations in the DBD appear to be more common in that malignancy (17). Sequencing of STAT6 in the extended set of 27 untreated DLBCLs and 22 Tly revealed a less focal pattern of mutations. B, representative images of nuclear phospho-STAT6 protein expression of STAT6 wild-type and STAT6 mutant GCB-type rDLBCL show that the KEGG-annotated JAK/STAT pathway is enriched in STAT6 mutants. The heatmap shows genes in the KEGG JAK/STAT pathway that are enriched across the exomes, thereby demonstrating value for using a combination of global and targeted techniques if sequencing were used to evaluate biomarkers of response in clinical trials.

Our data may inform on the design and interpretation of clinical trials studying the activity of targeted therapies. The frequencies of MYD88 and CD79B mutations in our ABC rDLBCLs were similar to those obtained by Wilson and colleagues, supporting that our genomic data is representative of other rDLBCL patients undergoing clinical trials (39). One important finding in our study is that deep sequencing of diagnostic biopsies can miss mutations that become clonally enriched at relapse, providing a rationale for obtaining another biopsy at the time of progression, especially in patients enrolling in clinical trials. Specifically, we show that many mutations involved in regulating NF-κB signaling can become clonally enriched at relapse (Table 2). The most commonly mutated genes involved were CD79B and MYD88, a pattern that is similar to de novo DLBCL. In addition, two regulators of NF-κB signaling, NFKBIE and NFKBIZ, which were under-represented in de novo DLBCL, may play a role in approximately 18% of rDLBCL. Thus, testing for these mutations in patients treated with NF-κB–targeted therapies, especially in clinical trials, may be indicated. Another important observation is that we provide insights into the biology of GCB-rDLBCL, a COO subtype in need of novel therapies and that represents half of the patients with rDLBCL entering clinical trials (40–42). This is counterintuitive because one might expect patients with the ABC subtype to be over-represented at the time of relapse, given their poor prognosis with RCHOP. We also demonstrate that the mutation spectrum of rGCB-DLBCL and rTly are very similar after therapy and generally distinguishable from ABC-rDLBCL, providing a rationale for including patients with rTly on more rDLBCL clinical trials, especially those investigating emerging therapeutics that specifically target GCB-associated pathways, for example, inhibitors of EZH2 activity (43).

This study prioritizes genes for future research focused on understanding therapeutic resistance in DLBCL. Our work directly complements previous studies of rDLBCL that were underpowered to detect mutations that were significantly more common at relapse (19, 20). TP53 emerged as a top candidate in our analyses, an expected result that supports our analytic approach. TP53 mutations are associated with RCHOP resistance and are selected for by DNA-damaging agents (44, 45). The novelty here is that we demonstrate examples of mutant VAF for TP53 exceeding 50% in rDLBCL, suggesting a tendency towards loss of the wild-type allele under the selective pressure of immunochemotherapy. This has important therapeutic implications, demonstrating the necessity of treatments for rDLBCL that act independently of TP53. The increased frequency of FOXO1 mutations was also not surprising given their negative prognostic role in de novo DLBCL, but the
trend towards acquisition of such mutations by clonal expansions following treatment has not been reported (29). We have also identified genes known to play a role in other lymphoid malignancies as having a probable role in DLBCL relapse, namely STAT6, FAS, CCND3, and NFKBIE. In contrast, the notably lower frequency of mutations in TMEM30A, EP300, SGK1, CD58, MEF2B, B2M, and PRDM1 in rDLBCL relative to untreated cohorts, though not statistically significant, might suggest they are not associated with RCHOP resistance or may represent a more curable group of tumors.

The presence of STAT6 mutations in approximately 40% of patients with GCB-type rDLBCL and Tly promote JAK/STAT signaling as a novel therapeutic target for these patients. STAT6 mutations, including D419, have been mostly reported in PMBCL and follicular lymphoma (18, 21). Wild-type phospho-STAT6 protein is not expressed in de novo DLBCL, but is expressed in most cases of PMBCL and HL, two lymphoma subtypes that are associated with very high cure rates (21, 46). Thus, it is unlikely that wild-type STAT6 induces chemoresistance, but rather the mutated version of STAT6, specifically at residue D419 in the DNA-binding domain, which appears to be selected for after chemotherapy in rDLBCL. The oncogenic function of mutated STAT6 D419 has not been elucidated, but the increased nuclear localization of phospho-STAT6 D419, increased expression of some STAT6 target genes, and the selection for a mutation that changes an amino acid at a single site, D419, are all consistent with its role as an activating mutation that promotes the survival of rDLBCL and Tly (22). Supporting this, STAT6-D419-mutated PMBCL cell lines depend on STAT6 signaling for their survival and are more sensitive to Jak2 inhibitors compared with STAT6 wild-type PMBCL cell lines (23, 47). Other mechanisms of increased JAK/STAT signaling have been reported in diagnostic DLBCL samples, such as mutations in SOCS1 and STAT3 (48, 49). However, these occurred in less than 5% of our cohort, suggesting that JAK/STAT signaling at relapse is predominantly driven by STAT6 mutations and represents a new therapeutic target for patients with Tly and rr-GCB-DLBCL (48, 49). Given that Jak2 inhibitors are routinely used to treat JAK2-mutated myeloproliferative disorders, and they are active in STAT6-mutant PMBCL cell lines, they could be readily tested in the setting of STAT6-mutated rDLBCL in future clinical trials.

We provide a comprehensive genome analysis of rDLBCL samples acquired after therapy that is representative of the patient population enrolling in clinical trials and in whom novel targeted therapies would be considered. Our data confirm the work by Liang and colleagues who showed that clonal evolution occurs in DLBCL under the selection pressure of RCHOP and subsequent therapies, by showing clonal enrichment of one or more mutation in 11 of 12 paired samples (19). A repeat biopsy at the time of relapse would therefore be warranted if the tumor biology were guiding the choice of therapy. Although our data support clonal selection for mutations in more than 20 genes across these cases, TP53, STAT6, MLL3, CCND3, and FOXO1 each have the greatest support for having a role in therapeutic resistance owing to significantly greater prevalence in our cohort. Future work on larger cohorts and paired specimens is needed to further prioritize individual mutations in these genes that are clinically significant. Identifying genes that are not known to play a role in de novo DLBCL, but are recurrently mutated in rDLBCL, is also novel. These included FAS, STAT6, NFKBIZ, and NFKBIE. Our work has highlighted that STAT6 mutations correlate with the presence of phospho-STAT6 protein. This may be a biomarker of activated JAK/STAT signaling and a potential novel therapeutic opportunity for patients with GCB-rDLBCL and Tly, patients in whom targeted therapies are needed. Finally, we have shown that the combination of exome and targeted sequencing within the context of a clinical trial is feasible. This can be useful to identify biomarkers of response to tested therapies and importantly, provide a valuable opportunity to gain further insights into the mechanisms of RCHOP resistance in DLBCL.

Disclosure of Potential Conflicts of Interest

S.E. Assouline reports receiving commercial research grants from Novartis, speakers bureau honoraria from Janssen, and is a consultant/advisory board member for Lundbeck. N.A. Johnson reports receiving commercial research grants from Roche, speakers bureau honoraria from Roche Canada, and Seattle Genetics, and is a consultant/advisory board member for Abbvie, Gilead, Janssen, Lundbeck, and Roche. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Assouline, A. Mohajeri, R.L. Johnston, L. Chong, D. Formika, K. Bushell, T. Petrogiannis-Haliotis, B.M. Grande, C. Rousseau, R. Froment, M. Arsenault

Study supervision: R.D. Morin, S. Assouline, M. Arsenault, K.K. Mann, N.A. Johnson

Other (experimental work): A. Mohajeri

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