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

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Abstract

Purpose: Relapsed or refractory diffuse large B-cell lymphoma (rrDLBCL) 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 rrDLBCL 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 DLBCLs 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*, *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 DLBCL including two regulators of NF-κB: *NFKBIE* and *NFKBIZ*. We detected mutations that may be affect sensitivity to novel therapeutics, such as *MYD88* and *CD79B* mutations, in 31% and 23% of patients with activated B-cell–type of rrDLBCL, respectively. We also identified recurrent *STAT6* mutations affecting D419 in 36% of patients with the germinal center B (GCB) cell rrDLBCL. 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 rrDLBCL and has identified novel therapeutic opportunities especially for the high-risk patients with GCB-type rrDLBCL. *Clin Cancer Res; 22(9); 2290–300. ©2015 AACR.*

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). DLBCL can be classified into two cell-of-origin (COO) molecular subtypes, the germinal center B-cell (GCB) type and activated B-cell (ABC) type, where the latter is associated with an inferior outcome (2, 3). DLBCL 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 DLBCL (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).

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Translational Relevance

Despite an improved understanding of the biology of *de novo* diffuse large B-cell lymphoma (DLBCL), the outcome of patients with relapsed and refractory (rrDLBCL) is still poor. Response rates to novel therapies that target specific pathways may depend on the tumor's mutation profile, which is unknown for patients with rrDLBCL at the time of relapse. We identified genes that are recurrently mutated at a higher frequency at relapse compared with diagnosis. We also provide evidence for clonal expansion of some mutations using paired diagnostic-relapse samples taken from the same patients. These mutations should be studied further as potential biomarkers of resistance to RCHOP chemotherapy. Our results highlight that JAK/STAT and NF-κB pathways are commonly altered in rrDLBCL, nominating these as promising avenues for targeted therapeutics. Although NF-κB has been heavily pursued as a target in DLBCL, the relevance of the JAK/STAT pathway in rrDLBCL has not been appreciated.

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, rrDLBCL samples are rare because patients are not routinely rebiopsied at the time of relapse outside the context of a clinical trial. Given that very few patients survive rrDLBCL, 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 rrDLBCL 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 rrDLBCL 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 rrDLBCL and rrTLy, 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-rrDLBCL and rrTLy (22). With the exception of PMBCLs, this pathway has not previously been implicated in *de novo* DLBCL.

Materials and Methods

Sample acquisition

This study included three DLBCL cohorts stratified based on the timing of sample acquisition. The first "relapse" cohort consisted of 38 patients whose samples were obtained after at least one cycle of immunochemotherapy, of which 25 evolved from *de novo* DLBCL, and 13 were TLy. Cases were selected on the basis of availability of both tumor tissue and germline DNA. In 27 of 38 cases, samples were obtained prospectively from patients enrolled on the Quebec Clinical Research Organization in Cancer (QCROC-2) clinical trial NCT01238692 (QC2- identifiers). In the remaining 11 of 38, eight were analyzed in the context of another study focusing on DLBCL in the Chinese population (CH-identifiers; ref. 20), and three samples were from patients treated in Montreal, Quebec (MT- samples). In 12 of 38 patients, we had matched pairs, where one sample was taken at diagnosis before RCHOP and a second tumor sample was taken at relapse. The clinical details and the type of analysis performed on each sample are provided in Supplementary Table S1. The second "diagnostic" cohort consisted of a different set of 138 patients with *de novo* DLBCL, for which good quality WES or genome data was publically available for reanalysis (7–10). This was used as the "control" group representing diagnostic DLBCL. The third "pretreatment" cohort consisted of 49 patients selected on the basis of the availability of tissue of their diagnostic DLBCL ($n = 27$) or TLy ($n = 22$) taken prior to initiation of therapy. These cases were used as pretreatment "controls" for the targeted resequencing of *STAT6* and other genes of interest (Supplementary Table S2). The diagnosis of DLBCL was confirmed by histopathology review, using the 2008 World Health Organization criteria (24). All patients provided informed consent either for the clinical trial or for storage of samples in a databank. This project was approved by the research ethics boards at the Jewish General Hospital and is in accordance with the declaration of Helsinki.

Sample preparation for the QCROC-2 trial

Four core-needle biopsies were performed for the 27 patients with rrDLBCL enrolled on the clinical trial. One core was fixed in formalin and embedded in paraffin (FFPET) for protein detection by immunohistochemistry (IHC). The remaining three cores were pooled in RPMI medium and DLBCL cells were enriched using a magnetic bead–negative selection technique (human B-cell enrichment cocktail without CD43; Stem Cell technologies) as previously described (25). DNA and RNA from purified B cells and peripheral blood mononuclear cells were isolated using the AllPrep kit (Qiagen).

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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 observed in libraries with a significant number of overlapping reads. Known single-nucleotide polymorphisms were not filtered from the analysis because all tumors were analyzed with matched normal DNA. Exome data were separately analyzed for somatic copy number variation (CNV) and loss of heterozygosity (LOH) using TITAN (28).

To facilitate comparison of mutation prevalence between relapsed DLBCL and diagnostic tumors, we obtained the raw sequence data from 138 diagnostic DLBCL tumor/normal pairs from four published studies and reanalyzed these for single-nucleotide variants (SNV) and insertions or deletions (indels) using a similar approach (7–10, 20).

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 patients. 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 TLy samples obtained prior to chemotherapy.

Gene expression profiling and gene-set enrichment analysis

We 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 (LLMP 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 and 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 *PIM1* (38%), *MYD88* (31%), *MLL3* (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" DLBCL 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*, *MPEG1*, *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

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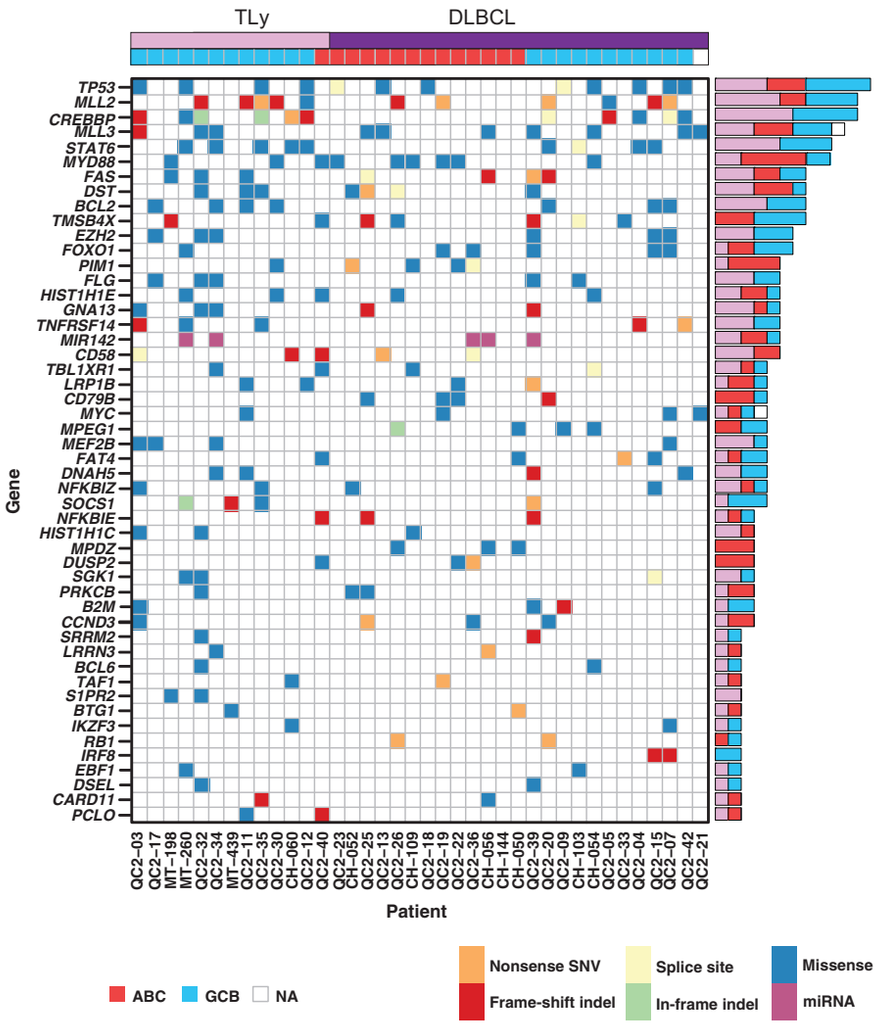

Figure 1.
Overview of somatic mutations identified in 38 rrDLBCLs and rrTLy. Mutations affecting genes previously identified as significant mutation targets in DLBCL and any additional genes found mutated in our cohort in at least three samples are shown. Each column corresponds to a sample where the patient identifiers are below and cases are ordered by the lymphoma type with rrTLy on the left (pink bar) and rrDLBCL on the right (purple bar). Cell of origin (COO) is indicated to designate GCB cases in blue and ABC cases in red. One case could not be assigned a COO and is shown in white. The type of coding mutation is indicated in the figure legend. Noncoding mutations are omitted with the exception of mutations affecting the miRNA gene MIR142 (purple), which were observed in multiple cases of rrDLBCL and rrTLy.

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

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

Gene mutated	% Mutations rrDLBCL $N = 25$[c]	% Mutations ABC $N = 13$	% Mutations GCB $N = 11$	% Mutations TLy $N = 13$	% Mutations Dx DLBCL $N = 138$
TP53[a]	32	23	45	31	13
MLL2	28	15	36	38	13
MLL3[a]	28	23	27	23	7
FOXO1[a,b]	24	15	36	8	9
CREBBP	20	0	45	46	13
MYD88	20	31	9	23	12
PIM1	20	38	0	8	14
STAT6[a]	16	0	36	38	4
FAS	16	15	18	23	7
CD79B	16	23	9	0	9
MPEG1[a]	16	15	18	8	3
BCL2	12	0	27	31	10
EZH2	12	0	27	23	13
CCND3[a]	12	15	9	8	1
MYC[a]	12	8	9	8	2
GNA13	8	8	9	23	14
CD58	8	15	0	23	7
NFKBIE	8	8	9	8	1
B2M	8	0	18	8	12
NFKBIZ[a]	8	8	9	15	0
TNFRSF14	8	0	18	23	8
MEF2B	4	0	9	23	8
SOCS1	4	0	9	15	1
CARD11	4	8	0	8	12

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: *MPEG1*: $P = 0.020$; *MLL3*: $P = 0.020$; *CCND3*: $P = 0.026$; *STAT6*: $P = 0.033$; *TP53*: $P = 0.033$; *NFKBIZ*: $P = 0.023$; 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*.

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 our series, we confirmed that these were somatic mutations because the variant allele was not present or < 1% in the germline. D419 mutations were restricted to GCB-rrDLBCL (36%) and rrTLy (38%), and were absent in ABC rrDLBCL (0%), further supporting that these were not polymorphisms but rather mutations associated with a specific tumor biology (Table 1). Because our analysis of paired biopsies indicated that *STAT6* mutations may exist subclonally (e.g., as low as 1%) in diagnostic samples, we performed deep sequencing of *STAT6* in an extended "pretreatment" cohort, consisting of *de novo* DLBCL and TLy samples taken prior to chemotherapy, to determine the incidence of clonal and subclonal *STAT6* mutations in diagnostic tumors. We observed coding mutations in 4

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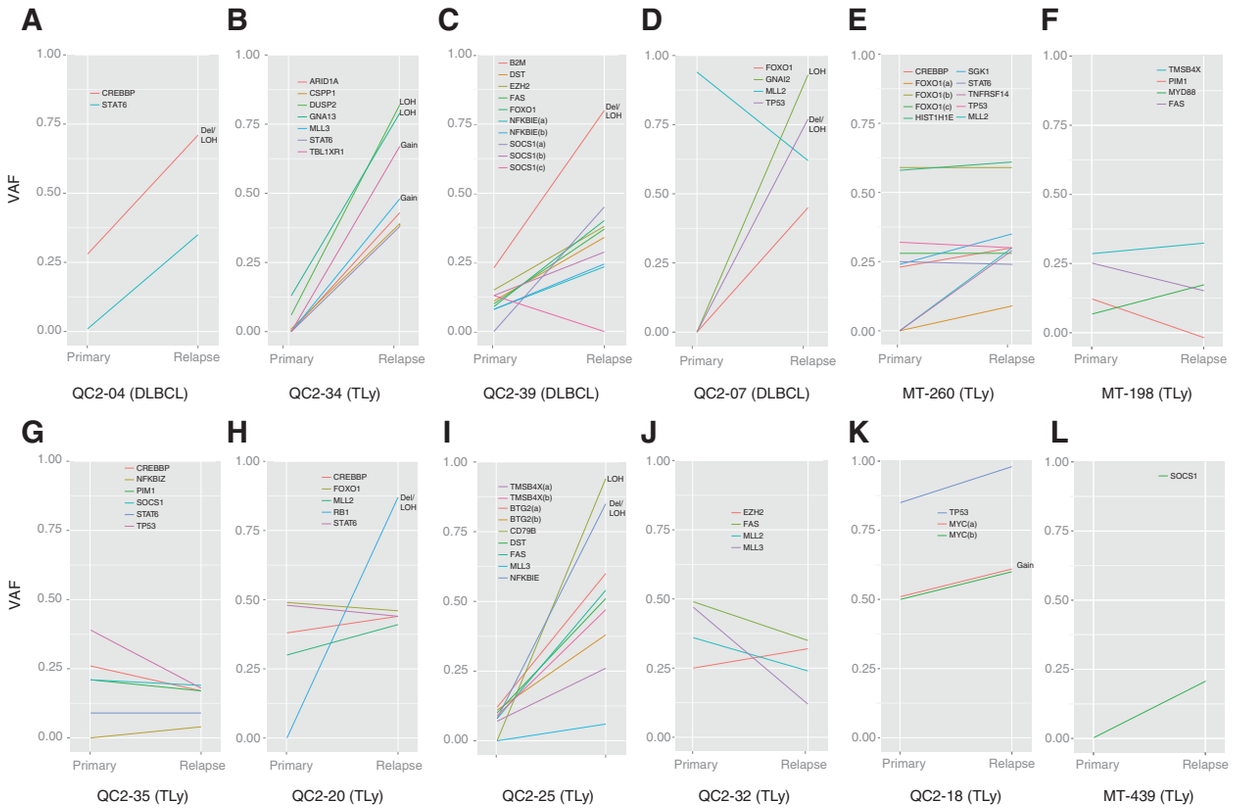

Figure 2.

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 (1%) 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*, *GNAI2* 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 *HIST1H1E* 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.

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).

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 FFPET 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

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

Sample	Gene	Chr	Position	T1 VAF	T2 VAF	Method (T1)	Method (T2)	Effect	AA change
MT-260	*FOXO1*	13	41239736	0.00	0.09	Amplicon	Amplicon	Missense	S205N
MT-260	*MLL2*	12	49422605	0.00	0.26	Amplicon	Exome	Splice site	—
MT-260	*TNFRSF14*	1	2488166	0.00	0.29	Amplicon	Exome	Frameshift deletion	—
MT-198	*MYD88*	3	38182641	0.09	0.20	Capture	Capture	Missense	L273P
MT-439	*SOCS1*	16	11349134	0.00	0.22	Capture	Capture	Frameshift deletion	—
QC2-04	*STAT6*	12	57496661	0.01	0.35	Amplicon	Amplicon	Missense	D419G
QC2-07	*FOXO1*	13	41240280	0.01	0.45	Amplicon	Capture	Missense	T24A
QC2-07	*GNAI2*	3	50289546	0.00	0.93	Amplicon	Amplicon	Missense	G45W
QC2-07	*TP53*	17	7577563	0.00	0.77	Amplicon	Exome	Missense	S240G
QC2-20	*MLL2*[a]	12	49425950	0.30	0.41	Amplicon	Exome	Nonsense	Q4180[b]
QC2-20	*CD79B*	17	62006660	0.00	0.36	Capture	Capture	Frameshift deletion	—
QC2-20	*CCND3*	6	41903688	0.00	0.35	Capture	Capture	Missense	I290R
QC2-20	*RB1*	13	48954198	0.01	0.77	Amplicon	Amplicon	Nonsense	R467[b]
QC2-25	*CD79B*	17	62006799	0.00	0.94	Amplicon	Amplicon	Missense	Y197D
QC2-25	*NFKBIE*	6	44232738	0.08	0.85	Amplicon	Capture	Frameshift deletion	—
QC2-32	*EZH2*[b]	7	148508727	0.25	0.32	Amplicon	Amplicon	Missense	Y646S
QC2-34	*EZH2*[b]	7	148508728	0.14	0.25	Capture	Capture	Missense	Y646N
QC2-34	*BCL2*[c]	18	60985727	0.00	0.38	Capture	Capture	Missense	H58L
QC2-34	*MEF2B*[c]	19	19260045	0.00	0.47	Capture	Capture	Missense	D83V
QC2-34	*ARID1A*	1	27105930	0.00	0.43	Amplicon	Amplicon	Frameshift insertion	—
QC2-34	*MLL3*	7	152012367	0.00	0.48	Amplicon	Amplicon	Missense	D149V
QC2-34	*CSPP1*	8	67976692	0.00	0.39	Amplicon	Amplicon	Missense	E20G
QC2-34	*TBL1XR1*	3	176767901	0.00	0.67	Amplicon	Exome	Missense	W196R
QC2-34	*STAT6*	12	57496662	0.00	0.43	Amplicon	Amplicon	Missense	D419N
QC2-35	*TNFRSF14*[b]	1	2489818	0.16	0.11	Capture	Capture	Missense	G72D
QC2-35	*CARD11*[b]	7	2983887	0.11	0.11	Capture	Capture	Frameshift Deletion	
QC2-35	*STAT6*[b]	12	57496662	0.17	0.12	Capture	Capture	Missense	D419H
QC2-35	*NFKBIZ*	3	101572431	0.00	0.04	Amplicon	Exome	Missense	A354D
QC2-39	*B2M*	15	45003746	0.23	0.80	Amplicon	Capture	Missense	M2R
QC2-39	*FOXO1*	13	41240015	0.09	0.40	Amplicon	Capture	Missense	G335A
QC2-39	*NFKBIE*[b]	6	44229483	0.08	0.23	Amplicon	Capture	Frameshift insertion	—
QC2-39	*SOCS1*	16	11349004	0.00	0.45	Amplicon	Capture	Missense	C332Y

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.

[a]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.

[b]VAF change was not reproduced by a targeted capture-based sequencing of this sample (Supplementary Table S6).

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

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 (Fig. 3C) demonstrates 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 GCB-rrDLBCL (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 rrDLBCLs 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 rrDLBCL to date and to our knowledge, the first time that exome sequencing was performed in rrDLBCL 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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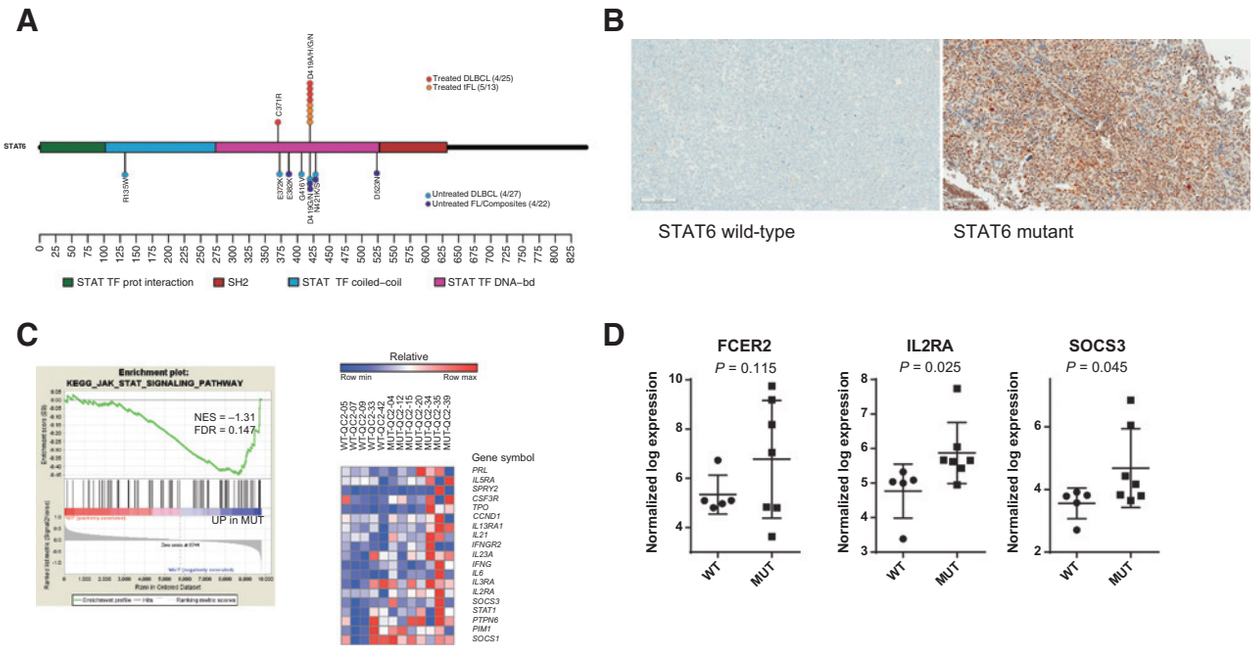

Figure 3.

Activation of the JAK/STAT pathway in *STAT6* and *SOCS1*-mutant lymphoma cells. A, in all but one of the *STAT6*-mutated rrDLBCL 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 rrDLBCL and STAT6 mutant rrDLBCL. C, gene-set enrichment analysis comparing STAT6 wild-type and STAT6 mutant GCB-type rrDLBC 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 in the mutant samples. D, mutant STAT6 target mRNAs (*FCER2*, *IL2RA*, and *SOCS3*) are increased in lymphomas harboring mutations in *STAT6* or *SOCS1*.

candidate genes based on prior knowledge of DLBCL, for example, recurrent indels in *NFKBIE* and missense mutations in *NFKBIZ*. We note that reliance solely on exome sequencing can yield insufficient sequence coverage for some genes, as illustrated by the low yield of *FOXO1* mutations 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 rrDLBCLs were similar to those obtained by Wilson and colleagues, supporting that our genomic data is representative of other rrDLBCL 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 rrDLBCL. 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-rrDLBCL, a COO subtype in need of novel therapies and that represents half of the patients with rrDLBCL 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 rrGCB-DLBCL and rrTLy are very similar after therapy and generally distinguishable from ABC-rrDLBCL, providing a rationale for including patients with rrTLy on more rrDLBCL 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 rrDLBCL 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 rrDLBCL, 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 rrDLBCL 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

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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 rrDLBCL 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 rrDLBCL 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 rrDLBCL. 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 rrDLBCL 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 rrDLBCL in future clinical trials.

We provide a comprehensive genome analysis of rrDLBCL 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 Jiang 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 rrDLBCL, 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-rrDLBCL 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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