

Specific Mutation Predict Relapse/Refractory Diffuse Large B-Cell Lymphoma

Jing Wang^{1,*}, Lei Tian^{2,*}, Weilong Zhang^{1,*}, Shuhan Tang¹, Wei Zhao¹, Yu Guo¹, Chaoling Wu¹, Yuansheng Lin¹, Xiaoyan Ke¹, Hongmei Jing¹

¹Department of Hematology, Lymphoma Research Center, Peking University Third Hospital, Beijing, 100191, People's Republic of China; ²Health Management Center, Peking University Third Hospital, Beijing, 100191, People's Republic of China

*These authors contributed equally to this work

Correspondence: Xiaoyan Ke; Hongmei Jing, Email xykbysy@163.com; hongmeijing@bjmu.edu.cn

Background: The application of rituximab has significantly enhanced the overall survival rates in patients with diffuse large B-cell lymphoma (DLBCL). Regrettably, a significant number of patients still progress to relapse/refractory DLBCL (rrDLBCL).

Methods: Herein, we employed targeted sequencing of 55 genes to investigate if gene mutations could predict the progression to rrDLBCL. Additionally, we compared the mutation profiles at the time of DLBCL diagnosis with those found in rrDLBCL cases.

Results: Our findings highlighted significantly elevated mutation frequencies of *TP53*, *MEF2B* and *CD58* in diagnostic biopsies from patients who progressed to relapse or refractory disease, with *CD58* mutations exclusively observed in the rrDLBCL group. In assessing the predictive power of mutation profiles for treatment responses in primary DLBCL patients, we found that the frequency of *CARD11* mutations was substantially higher in non-response group as compared with those who responded to immunochemotherapy. In addition, we revealed mutations in *HIST2H2AB*, *BCL2*, *NRXN3*, *FOXO1*, *HIST1H1C*, *LYN* and *TBL1XR1* genes were only detected in initial diagnostic biopsies, mutations in the *EBF1* gene were solely detected in the rrDLBCL patients.

Conclusion: Collectively, this study elucidates some of the genetic mechanisms contributing to the progression of rrDLBCL and suggests that the presence of *CD58* mutations might serve as a powerful predictive marker for relapse/refractory outcomes in primary DLBCL patients.

Keywords: targeted sequencing, mutation profile, relapse/refractory disease, diffuse large B-cell lymphoma

Introduction

Diffuse large B cell lymphoma (DLBCL) represents the most prevalent form of non-Hodgkin lymphoma (NHL), which accounts for 25–30% of all NHL cases with an annual incidence rate of 5.6 per 100,000 individuals.^{1,2} According to the cell of origin (COO) classification, DLBCL is divided into germinal center B-cell (GCB) and activated B-cell (ABC) subtypes. These subtypes exhibit distinct clinical outcomes, with the ABC subtype associated with an inferior prognosis.³ The standard first-line treatment for DLBCL comprises a combination of cyclophosphamide, doxorubicin, vincristine and prednisolone, augmented by rituximab (R-CHOP) immunochemotherapy. Although the incorporation of rituximab has significantly improved the prognosis of DLBCL patients, it has also led to the emergence of specific resistance mechanisms.⁴ About 40% of patients with lymphoma eventually develop a relapse or refractory status.⁵ The prognosis for patients with relapse or refractory DLBCL (rrDLBCL) remains inferior.⁶ Approximately 50% of patients with rrDLBCL achieve a response to second-line chemotherapy, and about half may undergo autologous hematopoietic stem-cell transplantation in certain clinical settings. Despite these interventions, 60% to 70% of these patients experience disease progression within 3 years following transplantation.^{6–8} Moreover, the median survival time for individuals with primary and refractory DLBCL is brief, ranging from only 5–7 months.⁶ Thus, elucidating the mechanisms driving the relapse or refractory progression of DLBCL is of critical importance.

Up to now, several studies have investigated the mutation profiles of primary central nervous system lymphoma (PCNSL) and rrDLBCL. For instance, Morin et al⁹ explored the genetic landscapes of rrDLBCL and identified *TP53*, *FOXO1*, *MLL3*, *CCND3*, *NFKBIZ* and *STAT6* as key candidate genes associated with therapeutic resistance. Similarly, Greenawalt et al¹⁰ demonstrated that mutations in *CREBBP* and *BCL2* genes were more prevalent in rrDLBCL compared to the diagnostic biopsies. Rushton et al¹¹ found 6 genes, namely *KMT2D*, *TP53*, *CREBBP*, *FOXO1*, *NFKBIE* and *MS4A1* (CD20), showed significant mutation enrichment at relapse in the circulating tumor DNA (ctDNA) samples of rrDLBCL compared with an independent primary cohort. However, there is a scarcity of research focusing on the mutation characteristics in the diagnostic biopsies that can predict the relapse/refractory outcomes of DLBCL.

In this study, we aimed to determine whether the mutation profiles can serve as predictive markers for relapse/refractory outcomes of DLBCL. Additionally, we compared the mutation profiles observed at the time of DLBCL diagnosis with those found in rrDLBCL patients.

Materials and Methods

Patient Information

This study included 96 lymph node samples from 64 primary DLBCL (before treatment) and 32 recurrence DLBCL patients (after treatment). Among these, 2 patients had both primary and relapse samples. The samples were collected from November 2011 to January 2021 at the Peking University Third Hospital (Beijing, China). Primary refractory DLBCL was defined as disease progression during initial R-CHOP regimen without achieving a complete remission (CR) or relapse within 6 months after a transient CR post-initial therapy. Relapsed DLBCL was defined as DLBCL that reappeared after a CR lasting more than 6 months. For comparison purposes, the 64 primary DLBCL patients were divided into DLBCL group (n=46) and rrDLBCL group (n=18) according to the late progression. Additionally, they were categorized into the response group (n=14) and non-response group (n=50). All patients received initial immunochemotherapy with or without radiotherapy or stem cell transplantation. This study got the approval of the Institutional Review Board of the Peking University Third Hospital Medical Science Research Ethics Committee (no. LM2020220) and was carried out in accordance with the Declaration of Helsinki.

Targeted Panel Sequencing

Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) or fresh tissues using the Maxwell[®] RSC DNA FFPE kit (Promega, Madison, Wisconsin, USA), referring to the manufacturer's descriptions. Targeted panel sequencing, encompassing 55 genes related to hematological malignancies ([Supplementary Table 1](#)), was performed on a NovaSeq platform (Illumina, San Diego, CA, USA). DNA quantity was determined using a Nanodrop 8000 UV-Vis spectrometer (NanoDrop Technologies, Wilmington, DE, USA), Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), and 2200 TapeStation Instrument (Agilent Technologies, Santa Clara, CA, USA). Library was constructed by lymphoma-associated gene mutation detection kit (Shanghai Rightongene Biotechnology Co., Ltd, Shanghai, China). The paired-end reads were aligned to the Human Genome Reference Consortium build 37 (GRCh37) using BWA (version 0.5.9-tpx). Samtools (v0.1.18), picard (v1.93) and GATK (v4.1.4.0) were used for BAM file handling, local realignment, base recalibration and calling variants, respectively. Mutations in the coding region were annotated using the Annovar software (version 2017-07-17).

Mutation Analysis

Variants, including single nucleotide variations (SNVs) and insertions/deletions (Indels), were screened by Shanghai Rightongene Biotechnology Co., Ltd. (Shanghai, China) based on the following filtering conditions: (1) SNVs or Indels with a mutation allele frequency (MAF) ≥ 0.001 in databases of 1000 genomes project,¹² 1000 genome East Asian, ExAC all or ExAC East Asian and genomAD¹³ were removed; (2) SNVs or Indels with a variant allele frequency (VAF) $\geq 5\%$ were retained; (3) variants listed in dbSNP (v147) and existing in the COSMIC database were retained; (4) SNPs or Indels including stopgain, stoploss, frameshift, nonframeshift and splicing site alterations were retained; (5) missense mutations meeting the following criteria were retained: SIFT score ≤ 0.05 , Polyphen2_HVAR_pred score ≥ 0.447 and

CADD score > 4. “Maftools” package (version 2.2.10) of the R software¹⁴ was used to generate the horizontal histogram illustrating the mutated genes.

Statistical Analysis

The differences in age, sex, COO classification, clinical stage, IPI (international prognostic index), LDH (lactate dehydrogenase) level, ALB (albumin) level, β -macroglobulin, HGB (hemoglobin) level, ESR (erythrocyte sedimentation rate) level, Ca⁺ level and DPL (double-protein-expression lymphomas) between the two groups were analyzed using Fisher’s exact tests. Kaplan-Meier (K-M) curves with Log rank tests were used to analyze the relationship between the mutations and the overall survival of DLBCL patients. Multivariate logistic regression analysis was conducted to assess the factors influencing the response to immunochemotherapy, with a p-value < 0.05 in univariate analysis serving as the threshold for inclusion. p-value < 0.05 was thought as significant difference.

Results

Mutation Characteristics of the Diagnostic Biopsies from Patients Who Developed to rrDLBCL

First, we described the mutation profiles of primary DLBCL patients, who were further divided into rrDLBCL and DLBCL groups, to explore whether certain mutation characteristics could predict relapse or refractory status of DLBCL. As shown in Table 1, the Ann Arbor stage and IPI in the rrDLBCL group were significantly elevated compared with the DLBCL group. Also, we compared the mutation profiles between these groups (Figure 1). The results showed significant differences in the mutation frequencies of *TP53*, *MEF2B* and *CD58*, especially noting that *CD58* mutations were exclusively detected in the rrDLBCL group (Figure 2A). However, no significant relationship was found between the mutations in *TP53*, *MEF2B*, *CD58* and the overall survival of DLBCL patients

Table 1 Clinicopathologic Features of Primary DLBCL Patients with Later Progression of rrDLBCL

Clinicopathologic Features	DLBCL (n=46)	rrDLBCL (n=18)	p
Age			0.417
<60	26	8	
≥60	20	10	
Gender			1
Male	23	9	
Female	23	9	
COO classification			0.24
GCB	10	1	
Non-GCB	36	17	
Ann Arbor Staging			0.044
I–II	19	2	
III–IV	27	16	
LDH level (mean±SD)	378.46±480.99	334.72±128.67	0.706

(Continued)

Table 1 (Continued).

Clinicopathologic Features	DLBCL (n=46)	rrDLBCL (n=18)	p
IPI			0.021
0–1	21	2	
2–3	19	10	
4–5	6	6	
ALB (mean±SD)	41.22±5.43	39.73±5.35	0.327
β-macroglobulin (mean±SD)	2.98±2.13	3.35±1.24	0.486
HGB (mean±SD)	123.02±16.27	117.22±19.07	0.227
ESR (mean±SD)	22.76±19.56	26.06±17.29	0.534
Ca+ (mean±SD)	2.33±0.17	2.27±0.24	0.199
DPL			0.268
Yes	19	4	
No	23	12	

Abbreviations: IPI, international prognostic index; LDH, lactate dehydrogenase; ALB, albumin; HGB, hemoglobin; ESR, erythrocyte sedimentation rate; DPL, double-protein-expression lymphomas; SD, standard deviation.

(Figure 2B–D), with a median follow-up time of (22.3 ± 2.2) months. These results indicated that *CD58* mutation might be an effective predictor of relapse or refractory status in primary DLBCL patients at the time of diagnosis.

Value of the Primary Mutation Profiles in Predicting the Curative Effect of DLBCL Patients in Response to Immunochemotherapy

In addition, we assessed the predictive value of the primary mutation profiles for determining the curative effect of immunochemotherapy in DLBCL patients. All patients in non-response group were diagnosed at stage III–IV, a significantly higher stage compared with the response group ($p=0.008$) (Table 2). In addition, the proportion of high-risk patients, as determined by the IPI, was significantly higher in the non-response group as compared with the response group ($p=0.002$) (Table 2). The mutation profiles showed that the frequency of *CARD11* mutations was significantly higher in non-response group as compared with the response group (Figures 3 and 4A). Multivariate analysis identified high-risk IPI (scores 4–5) ($p=0.017$) and *CARD11* mutation ($p=0.030$) as two independent variables influencing the response of patients to immunochemotherapy (Figure 4B).

Longitudinal Monitoring of the Mutation Profiles of DLBCL Relapse

Luckily, primary and recurrent samples from two DLBCL patients were included in this study. To longitudinally monitor the mutation profiles of DLBCL relapse, we assessed the changes in Variant Allele Frequency (VAF) in the 2 cases. In case 1, the VAFs of *PRDM1* and *CD79B* increased in relapse sample as compared with the primary sample (Figure 4C). In case 2, the VAFs of *DUSP2* (dual specificity phosphatase 2) (NM_004418.3:exon3:c.719T>C:p.Ile240Thr), *STAT3* and *BTG2* increased in the relapse sample, while the VAFs of *SGK1* and another *DUSP2* variant (NM_004418.3:exon3:c.730+1G>C) decreased (Figure 4D). These results suggested that mutations with varying VAFs between primary and relapse conditions might contribute to the progression of DLBCL.

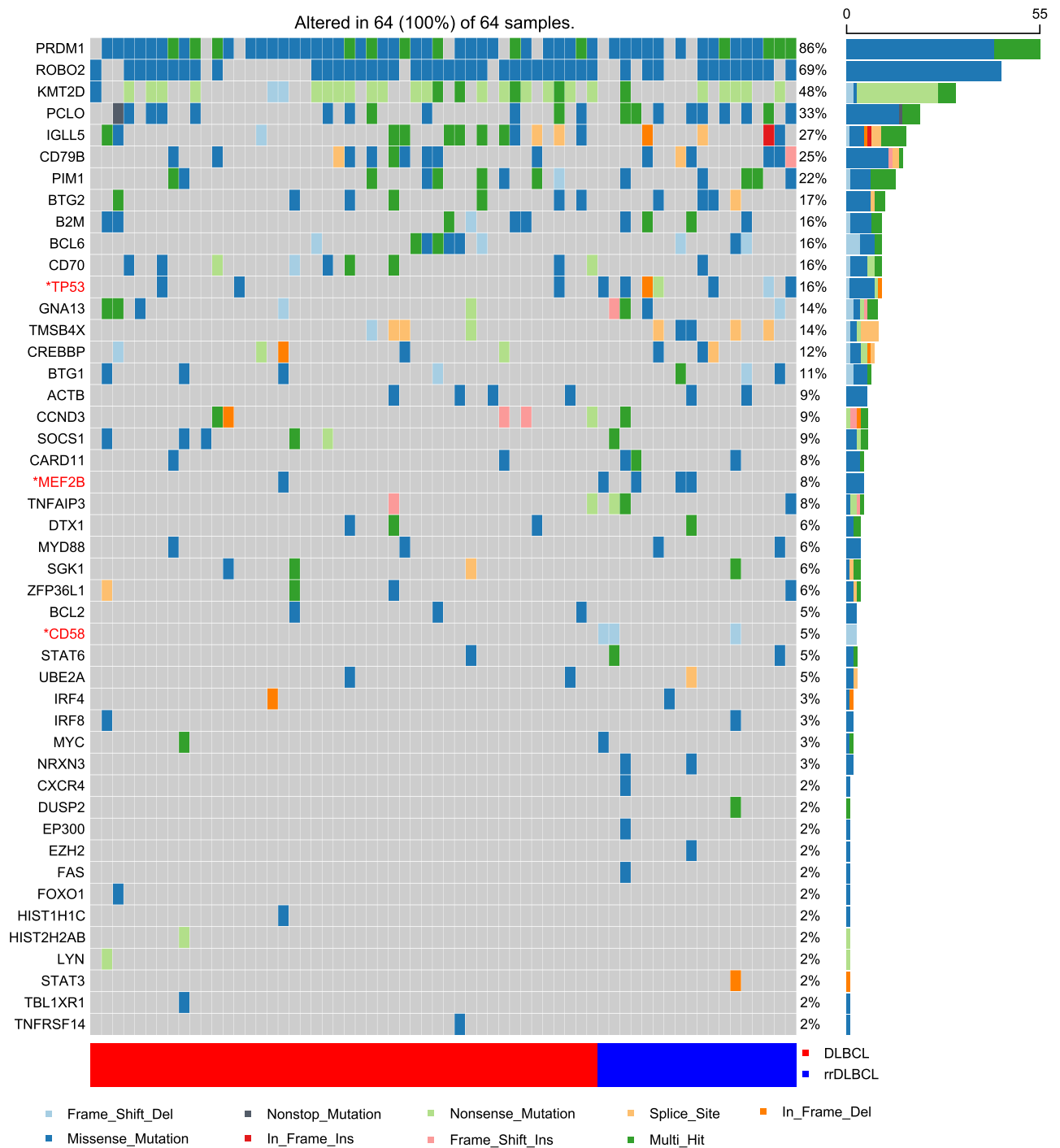


Figure 1 Comparison of mutation profiles in primary patients with and without progression to rrDLBCL. The horizontal histogram showed the mutation genes in the lymph node samples from primary patients who either progressed to or did not progress to rrDLBCL. * p value < 0.05, indicating the genes for which mutation frequency significantly differed between these two groups.

Horizontal Monitoring of Mutation Profiles in rrDLBCL

Also, we compared the mutation profiles of the biopsies from primary DLBCL patients and rrDLBCL patients. As shown in Figure 5, mutations in 7 genes (*HIST2H2AB*, *BCL2*, *NRXN3*, *FOXO1*, *HIST1H1C*, *LYN*, *TBL1XR1*) were detected exclusively in the primary DLBCL patients, while *EBF1* mutations was found only in the rrDLBCL biopsies. In addition, the frequency of *KMT2D* mutations was significantly lower in the rrDLBCL group (5/32) as compared with the primary

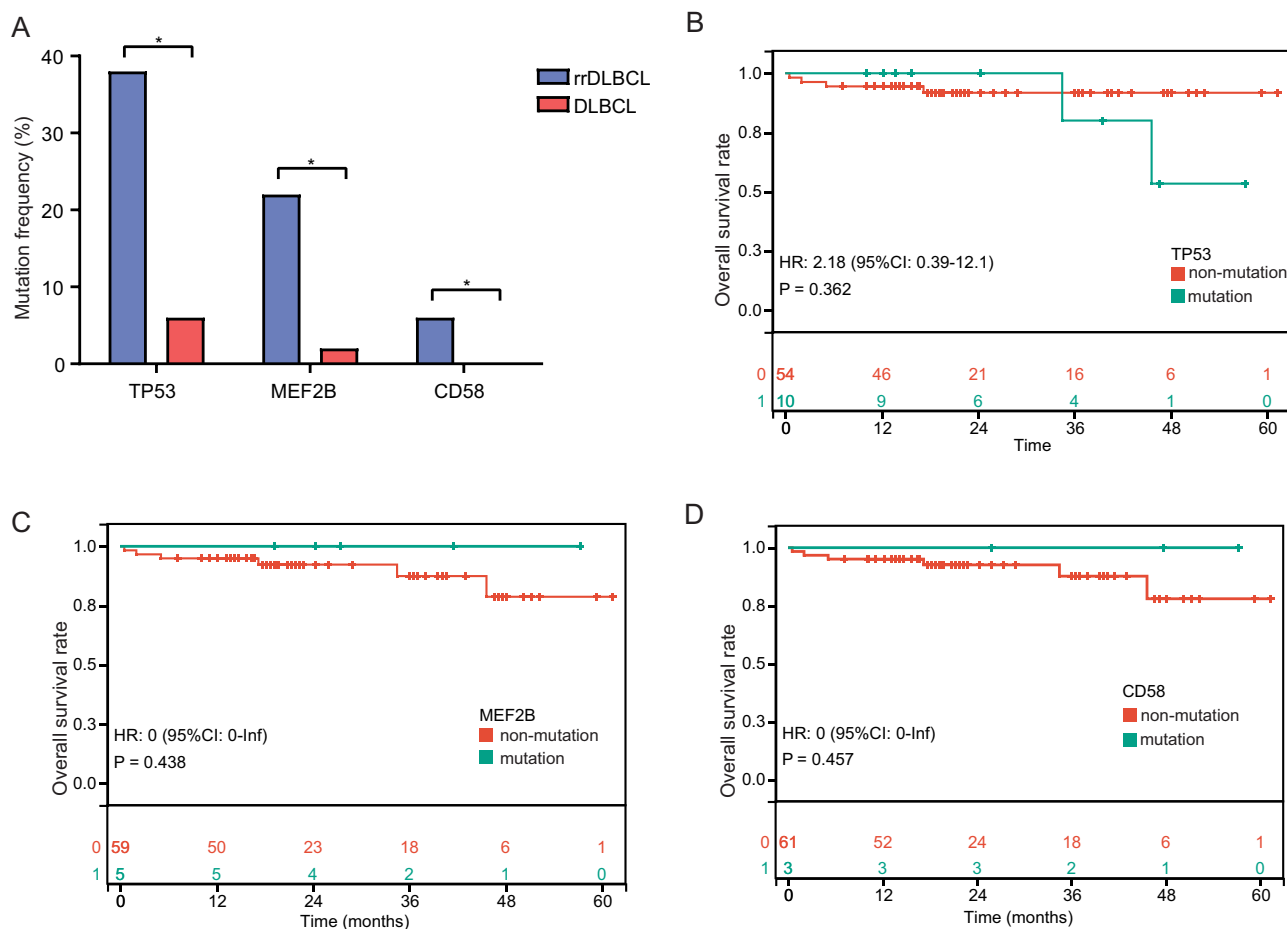


Figure 2 TP53, MEF2B and CD58 mutations and their prognostic implications in DLBCL. (A) Mutation frequencies of TP53, MEF2B and CD58 in diagnostic biopsies from DLBCL patients who proceeded to rrDLBCL versus those who did not (DLBCL group). (B–D) K-M curves depicting the relationship between the TP53, MEF2B and CD58 mutations and the overall survival in DLBCL patients.

DLBCL group (31/64). These results demonstrated that mutations in *HIST2H2AB*, *BCL2*, *NRXN3*, *FOXO1*, *HIST1H1C*, *LYN*, *TBL1XR1*, *EBF1*, and *KMT2D* might play roles in the progression of rrDLBCL.

Discussion

In this study, we evaluated the predictive value of mutation landscapes in predicting the relapse/refractory progression in DLBCL. From the targeted sequencing of 55 genes, we found that the mutation frequencies of *TP53*, *MEF2B* and *CD58* were significantly increased in the diagnostic biopsies of patients who later developed relapse or refractory disease. This

Table 2 Clinicopathologic Features of Primary DLBCL Patients with Later Response to Immunochemotherapy

Clinicopathologic Features	Response Group (n=50)	Non-response Group (n=14)	p
Age			0.546
<60	28	6	
≥60	22	8	

(Continued)

Table 2 (Continued).

Clinicopathologic Features	Response Group (n=50)	Non-response Group (n=14)	p
Gender			0.762
Male	24	8	
Female	26	6	
COO classification			1
0	9	2	
I	41	12	
Ann Arbor Stage			0.008
I-II	21	0	
III-IV	29	14	
LDH (mean±SD)	365.72±462.78	367.71±130.89	0.987
IPI			0.002
0-3	45	7	
4-5	5	7	
ALB (mean±SD)	41.26±5.41	39.16±5.28	0.203
β-microglobulin (mean±SD)	2.84±2.02	3.94±1.20	0.058
HGB (mean±SD)	123.66±16.74	113.29±16.68	0.044
ESR (mean±SD)	22.42±19.20	28.21±17.54	0.314
Ca2+ (mean±SD)	2.30±0.18	2.37±0.24	0.265
DPL			0.404
No	20	3	
Yes	26	9	

Abbreviations: IPI, international prognostic index; LDH, lactate dehydrogenase; ALB, albumin; HGB, hemoglobin; ESR, erythrocyte sedimentation rate; DPL, double-protein-expression lymphomas; SD, standard deviation.

finding is consistent with previous research that also highlighted the importance of specific genetic alterations in the progression of DLBCL.^{15,16} Mutations in *TP53*, a well-established anti-tumor and therapeutic resistant gene, have been reported in 20–25% of DLBCL cases, with a similar incidence in both GCB and ABC subtypes.^{9,11,17} Also, it has been demonstrated that *TP53* genes are more prevalent in rrDLBCL as compared with the primary DLBCL cases.¹⁸ In addition, accumulating evidence has demonstrated that *TP53* mutation serves as a negative prognostic factor in DLBCL.^{17,19,20} Targeting *TP53* mutation might represent a promising strategy for DLBCL patients harboring these mutations. Just so, several agents have been developed to reactivate functions of normal *TP53* in *TP53*-mutated tumor. These include molecules targeting a broad class of mutants to restore tumor suppressive functions (such as PRIMA, RITA, and scFv),^{21–23} and compounds specifically targeting missense mutations (like Phikan059 targeting R220C).²⁴ *MEF2B* (Myocyte enhancer-binding factor 2B) is an independent regulator of *BCL6* expression, a crucial master regulator in germinal center formation.^{25,26} *MEF2B* plays a significant role in regulating the proliferation of GC-derived lymphoma cells through partially modulating *BCL6* expression.²⁷ Notably, we found that CD58 mutation was

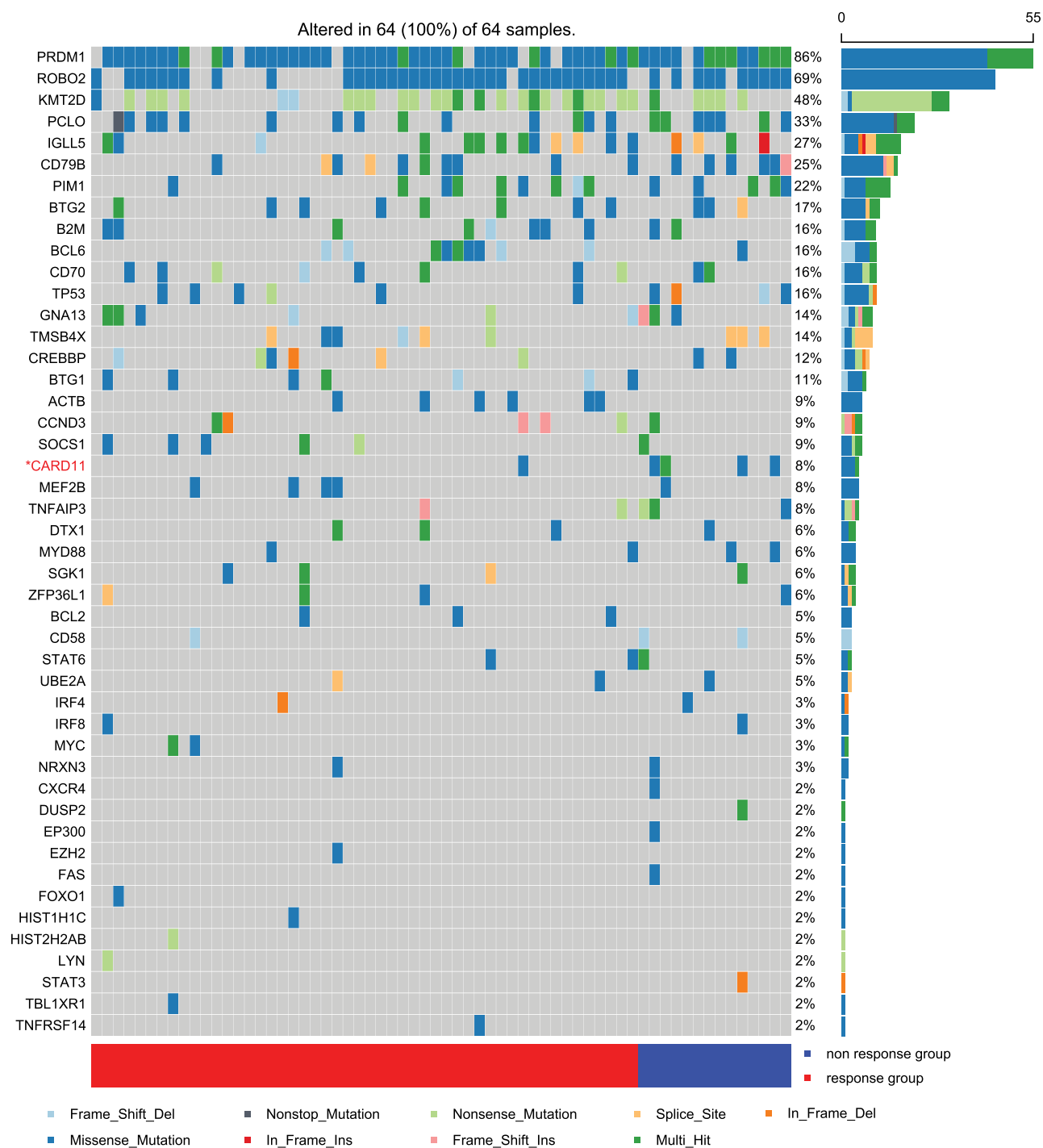


Figure 3 Comparison of mutation profiles in primary DLBCL patients based on response to immunochemotherapy. The horizontal histogram showed the mutation genes in the lymph node samples from primary patients who responded to the immunochemotherapy versus those who did not. * p value < 0.05 indicates genes for which mutation frequency significantly differed between responders and non-responders.

exclusively detected in the rDLBCL cohort compared to the DLBCL cohort. This finding aligns with previous research that reported a higher mutation frequency of CD58 in rDLBCL patients,²⁸ highlighting its potential as a potent marker for predicting relapse/refractory outcomes in primary DLBCL patients.¹⁶ CD58-coded protein is a member of the immunoglobulin superfamily, which is vital for tumor recognition through binding to CD2 expressed on T and NK cells.²⁹ Loss of CD58 expression has been identified to be associated with worse overall and event-free survival of

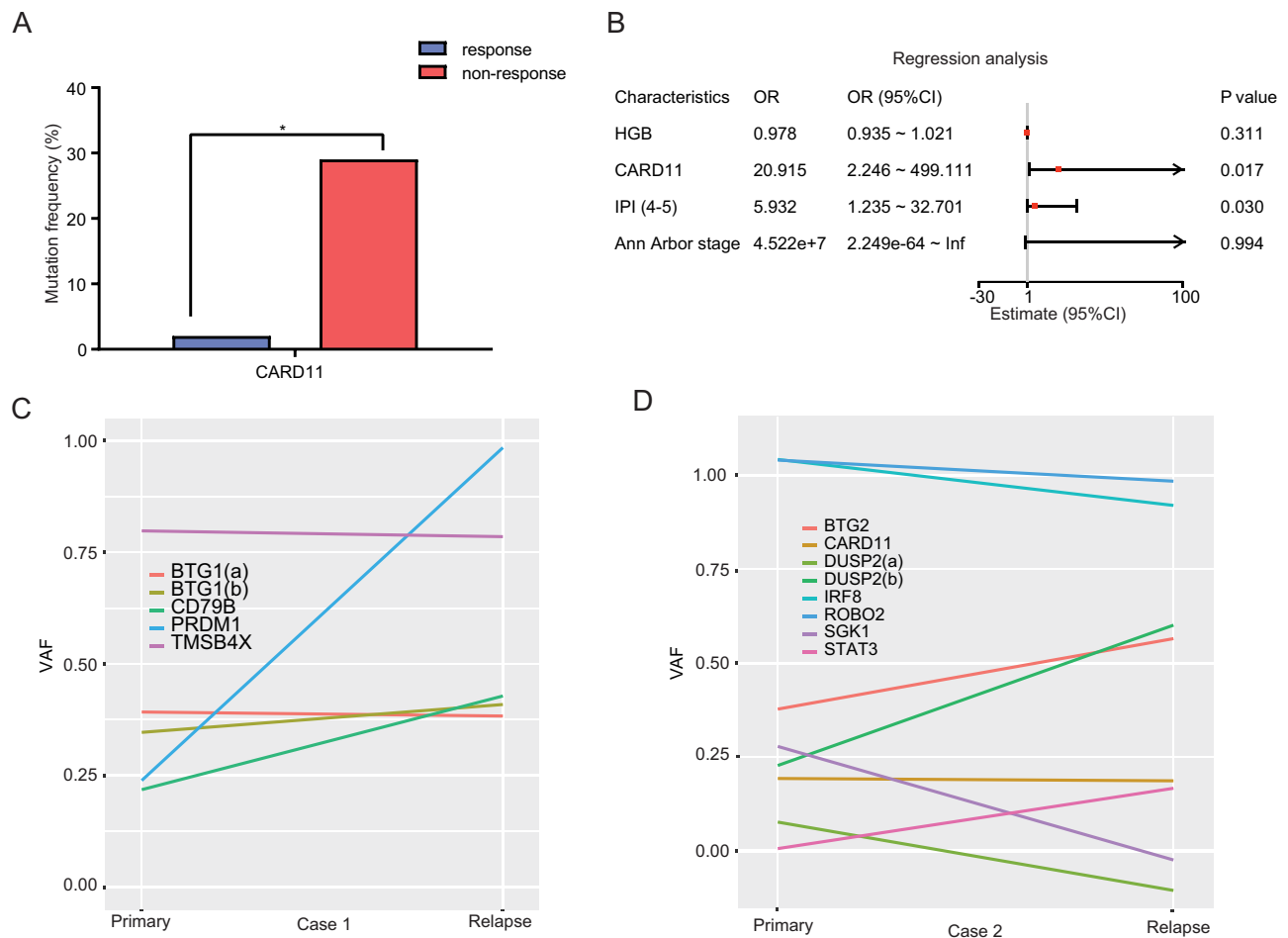


Figure 4 *CARD11* mutation status and Its Association with Immunochemotherapy Response and clonal evolution in DLBCL patients. **(A)** *CARD11* mutation frequencies in the diagnostic biopsies of DLBCL patients who responded to immunochemotherapy (response group) versus those who did not (non-response group). **(B)** Forest plot showing multivariate logistic analysis results to assess factors influencing the immunochemotherapy response in DLBCL patients. **(C and D)** Changes in the VAFs of mutated genes between the paired primary and relapsed DLBCL biopsies.

DLBCL patients.³⁰ Thus, we conjecture that restoring *CD58* expression may facilitate T and NK cell-mediated immune recognition to lymphoma cells, potentially increasing the efficacy of immunotherapy.

To further explore the value of mutations in predicting the relapse/refractory DLBCL, the primary DLBCL patients were divided into response group and non-response group based on the curative response to immunochemotherapy. Then, we compared the mutation profiles of the two groups. The results showed that the frequency of *CARD11* mutation was significantly higher in the non-response group as compared with the response group. This suggests that *CARD11* mutations may be associated with patients' response to immunochemotherapy, echoing the findings of studies exploring molecular mechanisms underlying lymphoma cell sensitivity to treatment, possibly focusing on epigenetics or the immune microenvironment.¹⁵ Also, multivariate logistic analysis showed that high-risk IPI (scores 4–5) and *CARD11* mutation were the two independent variables influencing patient response to immunochemotherapy. *CARD11* encodes a multi-adaptor or immune signaling protein essential for propagating signals in immune cells. Mutations in *CARD11* have been implicated in carcinogenesis. Gain-of-function variants act downstream of T- and B-cell receptors in lymphoid cells, leading to NF- κ B activation and promoting lymphogenesis.³¹ On the contrary, loss-of-function variants are linked to severe combined immunodeficiency (SCID)³² and combined immune deficiency.³³ In the current study, we found 6 missense variants of *CARD11* in 5 out of 64 cases with primary DLBCL. Previously, Lenz et al³⁴ detected the missense mutations of *CARD11* in 7 out of 73 ABC DLBCL biopsies, all within exons encoding the coiled-coil domain. Introducing *CARD11* coiled-coil domain mutations into lymphoma cells triggered constitutive NF- κ B activation.³⁴

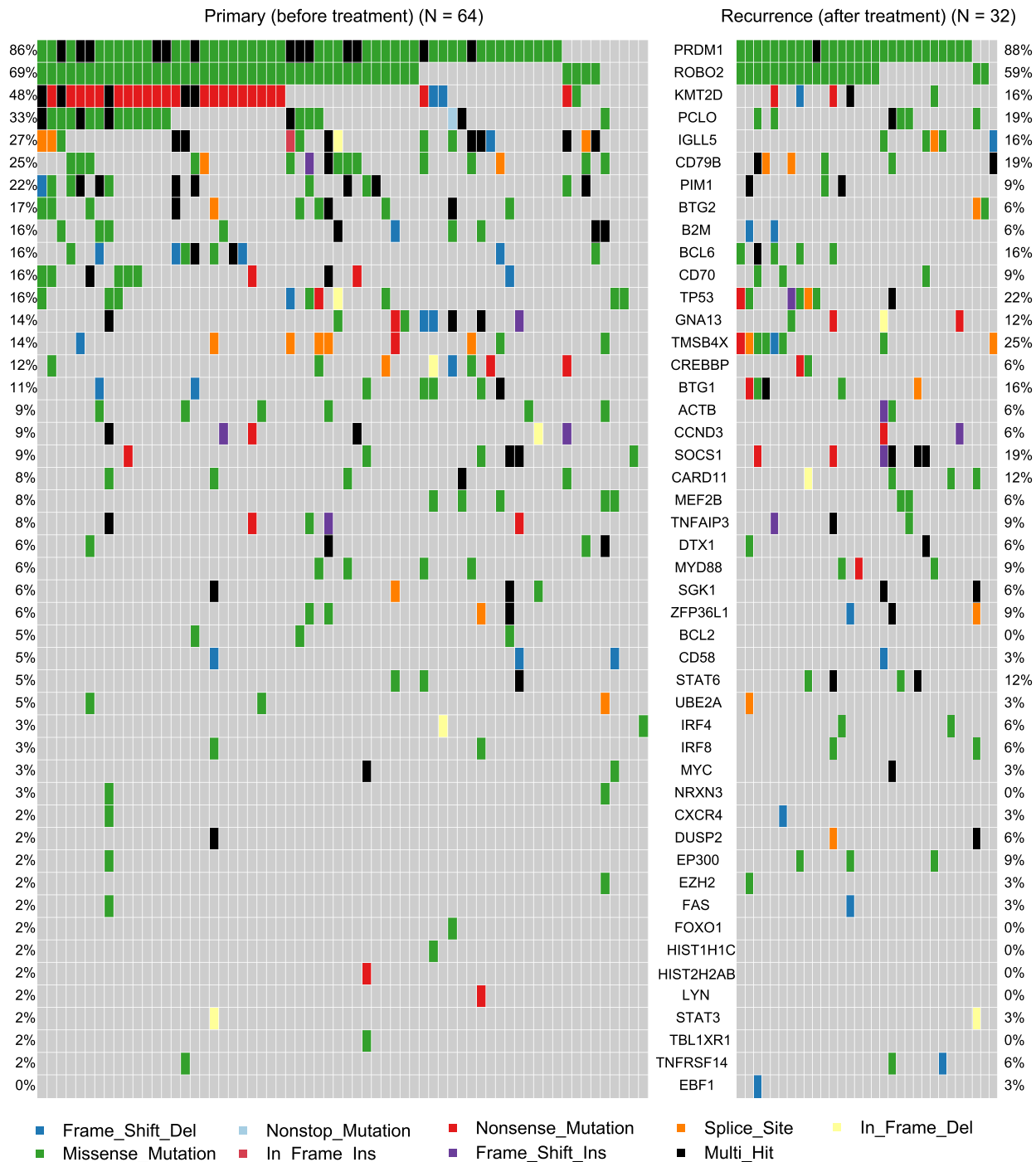


Figure 5 Mutation profiles in primary (pre-treatment) and recurrent (post-treatment) DLBCL patients. The horizontal histogram illustrates the differences in mutation profiles of lymph node samples between primary DLBCL and rrDLBCL patients.

Here, we found 4 *CARD11* variants located in exons encoding the coiled-coil domain and two in the linker domain. We infer the *CARD11* missense mutations may contribute to the resistance to immunochemotherapy through activating NF-κB signaling pathway.

In addition, we longitudinally evaluated the mutation profiles of DLBCL relapse in primary and relapse samples from two DLBCL patients. The integrated analysis showed that the VAFs of *PRDM1*, *CD79B*, *DUSP2* (NM_004418.3: exon3: c.719T>C: p.Ile240Thr), *STAT3* and *BTG2* mutants increased in relapse samples, while the VAFs of *SGK1* and *DUSP2*

(NM_004418.3:exon3:c.730+1G>C) decreased as compared with the diagnostic biopsies. *CD79B* was widely expressed on the tumor cells across various lymphomas regardless of stage, subtype, and cytogenetic and molecular features, suggesting that *CD79B* might serve as a potent target for CAR T-cell therapy of B-cell lymphomas.³⁵ Furthermore, we found variations in 7 genes (*HIST2H2AB*, *BCL2*, *NRXN3*, *FOXO1*, *HIST1H1C*, *LYN*, *TBLIXR1*) that were exclusively detected in primary DLBCL patients, while *EBF1* mutation was only detected in the rrDLBCL biopsies though horizontal monitoring of the mutation profiles in rrDLBCL. Using the Gene Expression Profiling Interactive Analysis database (GEPIA, <http://gepia.cancer-pku.cn/>), we found that the expression of *BCL2*, *FOXPI*, *TBLIXR1* and *EBF1* was significantly increased in DLBCL tissues as compared with normal tissues. Evidence has shown that three of these genes (*BCL2*, *FOXO1*, *TBLIXR1*) are involved in the NF- κ B pathway,^{36–38} and *EBF1* variants are common in DLBCL non-responders.³⁸

In this study, several limitations should be acknowledged. Firstly, the small sample size may affect the generalizability of the findings. Secondly, we did not conduct a comprehensive analysis of the gene expression levels associated with significantly different mutation frequencies. Therefore, further research is needed to verify our results.

Collectively, this study enhances our understanding of the mechanisms driving the progression of rrDLBCL and suggests that *CD58* mutation might serve as a valuable marker to predict the relapse/refractory outcomes in primary DLBCL patients.

Data Sharing Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

The studies involving human participants were reviewed and approved by the Ethics Committee of the Peking University Third Hospital. The patients/participants provided their written informed consent to participate in this study. Registry and the Registration No: N/A. Animal Studies: N/A.

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Disclosure

The authors declare no conflicts of interest in this study.

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