



Research Paper

B-cell Function Gene Mutations in Diffuse Large B-cell Lymphoma: A Retrospective Cohort Study



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ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous subtype of non-Hodgkin lymphoma. In addition to clinical and immunophenotypic characteristics, recurrent gene mutations have recently been identified in patients with DLBCL using next-generation sequencing technologies. The aim of this study is to investigate the clinical relevance of B-cell function gene mutations in DLBCL. Clinical analysis was performed on 680 Chinese DLBCL patients (146 non-CR and 534 CR cases) treated with six cycles of 21-day R-CHOP (Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone), alone or followed by two additional doses of rituximab consolidation on patients' own intention. Somatic mutations of B-cell function genes were further screened on 275 (71 non-CR and 204 CR) cases with available tumor samples by targeted sequencing, including genes involved in B-cell receptors (BCRs) pathway (*CARD11*, *LYN*, *CD79A*, and *CD79B*), Toll-like receptors (TLRs) pathway (*MYD88*), and tumor necrotic factor receptor (TNFR) pathway (*TRAF2* and *TNFAIP3*). B-cell function gene mutations occurred in 44.0% (121/275) of DLBCL patients. The TLRs and TNFR related gene mutations were more frequently observed in non-CR patients ($p = 0.019$ and $p = 0.032$, respectively). BCRs related gene mutations, as well as revised IPI (R-IPI) and double BCL-2/MYC expression, were independently related to short progression-free survival in DLBCL after CR. The adverse prognostic effect of BCRs related gene mutations could be overcome by two additional doses of rituximab consolidation. These results highlight the molecular heterogeneity of DLBCL and identify a significant role of B-cell function gene mutations on lymphoma progression and response to rituximab in DLBCL.

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1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous subtype of non-Hodgkin lymphoma with varied clinical, immunophenotypic and genetic features (SH, 2008). Although the outcome of DLBCL patients has been significantly improved by anti-CD20 monoclonal antibody rituximab combined with induction chemotherapy (mainly as cyclophosphamide, doxorubicin, vincristine, and prednisone [R-CHOP]); the lack of remission or early relapse remains a major clinical issue. Therefore, the identification of biomarkers related to therapeutic efficacy, particularly the response to rituximab, may be greatly helpful to conduct risk stratification treatment in DLBCL.

In the era of rituximab, in addition to clinical parameters based on International Prognostic Index (IPI) (Sehn et al., 2007), tumor cell of origin (COO) (Alizadeh et al., 2000), as well as BCL-2 and MYC double translocation/expression (Johnson et al., 2012; Green et al., 2012; Horn et al., 2013), are validated as important prognostic indicators of DLBCL. More recently, recurrent gene mutations have been revealed by next-generation sequencing technologies, including those involved in B-cell function (Compagno et al., 2009; Ngo et al., 2011; Davis et al., 2010; Lenz et al., 2008; Kheirallah et al., 2010). B-cell receptors (BCRs) activate NF- κ B by harboring receptor mutations in *CD79A* or *CD79B*, along with mutations of kinase *LYN* or effector *CARD11* (Rossi et al., 2013). Toll-like receptors (TLRs) recruit protein kinases mainly via adaptor molecule *MYD88*, also leading to NF- κ B activation (Rossi et al., 2013). As an important member of the tumor necrotic factor receptor (TNFR) associated factor (TRAF) protein family, *TRAF2* is responsible for TNF- α -mediated modulation of NF- κ B (Rossi et al., 2013). Finally, *TNFAIP3* mutations resulted in a loss of NF- κ B cascade inhibition (Honma et al., 2009). All these genes are closely associated with B-cell function and frequently mutated in DLBCL (Compagno et al., 2009).

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However, their relationship with lymphoma progression and treatment response warrants further investigation in DLBCL.

In the present study, we assessed the mutational pattern of key B-cell function genes on a large cohort of Chinese DLBCL patients treated with R-CHOP. The results showed that B-cell function gene mutations occurred in 44.0% of DLBCL patients, with the TLRs and TNFR related gene mutations reflecting non-remission status. Along with revised IPI (R-IPI) and double BCL-2/MYC expression, the presence of BCRs related gene mutations independently correlates with the disease relapse in DLBCL, which could be overcome by two additional doses of rituximab consolidation.

2. Methods

2.1. Patients

From December 2002 to December 2012, a total of 901 consecutive patients with de novo DLBCL based on registry data were enrolled in this study. The histological diagnosis was established according to World Health Organization (WHO) classification (SH, 2008), with exclusion of mediastinal large B-cell lymphoma or primary central nervous system DLBCL. A flow chart describing the cohort selection was outlined in Fig. 1. IPI (1993), R-IPI (Sehn et al., 2007) and National Comprehensive Cancer Network (NCCN)-IPI (Zhou et al., 2014) were calculated, as previously described. The study was approved by the Shanghai Rui Jin Hospital Review Board with informed consent obtained in accordance with the Declaration of Helsinki.

2.2. Response Criteria

The treatment response was evaluated according to the International Workshop Criteria (Cheson et al., 1999; Cheson et al., 2007). Patients with complete remission (CR) and unconfirmed complete remission (CRu) were defined as CR group, while patients with partial response or no response were defined as non-CR group.

2.3. Immunohistochemistry

Immunohistochemistry was performed on 5 μ m-paraffin sections with an indirect immunoperoxidase method using antibodies against CD10, BCL-6, MUM-1, Ki-67, BCL-2, MYC, NF- κ B1 (p105/p50, 1:250, Cell Signaling Technology) and NF- κ B2 (p100/p52, 1:300, Cell Signaling Technology). Germinal center B-cell (GCB) or non-GCB subgroups were determined using Hans classification (Hans et al., 2004), with 30% cut-

off value of CD10, BCL-6, and MUM-1. As for BCL-2/MYC double expression, cut-off value of BCL-2 and MYC were 70% and 40% respectively, as previously described (Hu et al., 2013). Nuclear NF- κ B localization for >30% of tumor cells was considered positive for NF- κ B activity (Compagno et al., 2009).

2.4. Targeted Sequencing

Genomic DNA was extracted from formalin-fixed paraffin-embedded tumor tissue and matched peripheral blood from patients with DLBCL, using a QIAamp DNA FFPE Tissue Kit (Qiagen) and a QuickGene DNA Whole Blood Kit L (Kurabo), respectively. Sequences for B-cell function genes, including *CD79A*, *CD79B*, *LYN*, *CARD11*, *MYD88*, *TRAF2* and *TNFAIP3* were obtained from the UCSC Human Genome database, using the corresponding mRNA accession number as a reference. PCR primers were designed by iPLEX Assay Design software (Sequenom), adding universal sequence tags (CS1 and CS2) to the targeted sequencing forward and reverse primers, which produce amplicons about 200 bp at the coding regions of the genes of interest. Microfluidic PCR reactions were run in a 48 \times 48 Access array system (Fluidigm) with FastStart High Fidelity PCR system (Roche) and high-throughput DNA sequencing was performed on Illumina Genome Analyzer Ix (GAIIx) and HiSeq2000 systems, according to the manufacturer's instructions. SAMtools version 0.1.19 was used to generate chromosomal coordinate-sorted bam files and to remove PCR duplications. Cases with identified mutations were sent for Sanger sequencing for verification. Primer sequences and polymerase chain reaction (PCR) conditions for each gene are available upon request. PCR reactions were run in a total volume of 25 μ l containing 1UGoTaq polymerase (Promega), 0.4 μ M of forward and reverse primers, 1.5 mM MgCl₂, 200 μ M dNTPs and 10 ng DNA.

2.5. Statistical Analysis

Baseline characteristics of patients were analyzed using two-sided χ^2 test. Progression-free survival (PFS) was calculated from the date when treatment began to the date when the disease progression was recognized or the date of the last follow-up. Overall survival (OS) time was measured from the date of diagnosis to the date of death or the last follow-up. Survival functions were estimated using the Kaplan-Meier method and compared by log-rank test. Univariate hazard estimates were generated with unadjusted Cox proportional hazards models. Covariates demonstrating significance with $p < 0.100$ on univariate analysis were included in the multivariate model. Statistical significance was defined as $p < 0.050$. All statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) 20.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Clinical and Pathological Characteristics

As shown in Fig. 1, 737 of 901 patients with DLBCL were scheduled to receive six cycles of 21-day R-CHOP as induction chemotherapy. Excluding 57 cases who discontinued treatment due to adverse events or patients' intention, and 146 cases who failed to achieve CR; 534 CR patients were either given additional two cycles of rituximab on a 21-day basis (AR, $n = 264$) or under observation (OBS, $n = 270$), on the principle of the patients' intention.

The main characteristics of the non-CR and CR patients were summarized in Table 1. Non-CR patients had multiple adverse prognostic factors of IPI, including age > 60 years (47.9% vs 33.0%, $p = 0.001$), poor performance status (25.3% vs 7.3%, $p < 0.001$), advanced Ann Arbor stage (74.0% vs 45.3%, $p < 0.001$), elevated serum lactic dehydrogenase (LDH) level (74.7% vs 36.0%, $p < 0.001$) and multiple extranodal involvement (34.2% vs 15.9%, $p < 0.001$), as compared to those of the CR

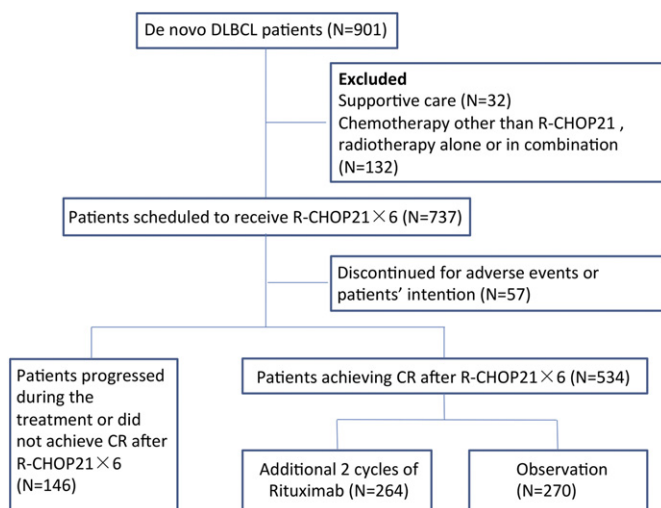


Fig. 1. Flow chart of the study. DLBCL: diffuse large B-cell lymphoma; R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone; CR: complete remission.

Table 1
Clinical and pathological characteristics of the patients with DLBCL.

Characteristics	Total (n = 680)	Non-CR (n = 146)	CR (N = 534)		p value ^a	p value ^b
			Additional rituximab (n = 264)	Observation (n = 270)		
Gender						
Male	384/680 (56.5%)	85/146 (58.2%)	155/264 (58.7%)	144/270 (53.3%)	0.223	0.639
Female	296/680 (43.5%)	61/146 (41.8%)	109/264 (41.3%)	126/270 (46.7%)		
Age (years)						
≤60	434/680 (63.8%)	76/146 (52.1%)	184/264 (69.7%)	174/270 (64.4%)	0.199	0.001
>60	246/680 (36.2%)	70/146 (47.9%)	80/264 (30.3%)	96/270 (35.6%)		
Performance status (ECOG)						
0–1	604/680 (88.8%)	109/146 (74.7%)	246/264 (92.2%)	249/270 (92.2%)	0.741	<0.001
≥2	76/680 (11.2%)	37/146 (25.3%)	18/264 (6.8%)	21/270 (7.8%)		
Ann Arbor Stage						
I–II	330/680 (48.5%)	38/146 (26.0%)	141/264 (53.4%)	151/270 (55.9%)	0.602	<0.001
III–IV	350/680 (51.5%)	108/146 (74.0%)	123/264 (46.6%)	119/270 (44.1%)		
Lactic dehydrogenase						
Normal	379/680 (55.7%)	37/146 (25.3%)	168/264 (63.6%)	174/270 (64.4%)	0.857	<0.001
Elevated	301/680 (44.3%)	109/146 (74.7%)	96/264 (36.4%)	96/270 (35.6%)		
Extranodal involvement						
0–1	545/680 (80.1%)	96/146 (65.8%)	219/264 (83.0%)	230/270 (85.2%)	0.554	<0.001
>1	135/680 (19.9%)	50/146 (34.2%)	45/264 (17.0%)	40/270 (14.8%)		
International Prognostic Index (IPI)						
Low	342/680 (50.3%)	30/146 (20.6%)	149/264 (56.4%)	163/270 (60.4%)	0.394	<0.001
Low-intermediate	167/680 (24.6%)	38/146 (26.0%)	63/264 (23.9%)	66/270 (24.4%)		
Intermediate-high	106/680 (15.6%)	44/146 (30.1%)	37/264 (14.0%)	25/270 (9.3%)		
High	65/680 (9.6%)	34/146 (23.3%)	15/264 (5.7%)	16/270 (5.9%)		
Revised International Prognostic Index (R-IPI)						
Very good	165/680 (24.3%)	9/146 (6.2%)	83/264 (31.4%)	73/270 (27.0%)	0.109	<0.001
Good	342/680 (50.3%)	57/146 (30.9%)	129/264 (48.9%)	156/270 (57.8%)		
Poor	173/680 (25.4%)	80/146 (54.8%)	52/264 (19.7%)	41/270 (15.2%)		
National Comprehensive Cancer Network (NCCN)-IPI						
Low	154/680 (22.6%)	14/146 (9.6%)	74/264 (28.0%)	66/270 (24.4%)	0.306	<0.001
Low-intermediate	325/680 (47.8%)	47/146 (32.2%)	128/264 (48.5%)	150/270 (55.6%)		
Intermediate-high	173/680 (25.4%)	69/146 (47.3%)	54/264 (20.5%)	50/270 (18.5%)		
High	28/680 (4.1%)	16/146 (11.0%)	8/264 (3.0%)	4/270 (1.5%)		
Cell of origin						
GCB	192/581 (33.0%)	36/123 (29.3%)	77/228 (33.8%)	79/230 (34.3%)	0.992	0.333
non-GCB	389/581 (67.0%)	87/123 (70.7%)	151/228 (66.2%)	151/230 (65.7%)		
Double BCL-2/MYC expression						
Positive	172/470 (36.6%)	49/108 (45.4%)	67/191 (35.1%)	56/171 (32.7%)	0.658	0.040
Negative	298/470 (63.4%)	59/108 (54.6%)	124/191 (64.9%)	115/171 (67.3%)		
Ki-67 > 80%						
Yes	263/517 (50.9%)	58/110 (52.7%)	113/223 (50.7%)	92/184 (50.0%)	0.921	0.669
No	254/517 (49.1%)	52/110 (47.3%)	110/223 (49.3%)	92/184 (50.0%)		

^a p value indicated difference between additional rituximab and observation.

^b p value indicated difference between non-CR and CR.

patients. Consequently, in terms of IPI, R-IPI and NCCN-IPI, 20.6%, 6.2% and 9.6% of the patients were categorized as low-risk or very good in the non-CR group, significantly lower than those of the CR group (58.4%, 29.2% and 26.2%, p all < 0.001). In the pathological setting, with similar distribution of GCB and non-GCB subtype, the non-CR patients showed significantly higher percentage of double BCL-2/MYC expression than the CR patients (45.4% vs 34.0%, $p = 0.040$).

Of note, according to the clinical and pathological characteristics, no obvious difference was observed between the AR and the OBS group of the CR patients (Table 1).

3.2. Mutational Pattern of B-cell Function Genes

Mutations of B-cell function genes were screened in 71 of the 146 non-CR patients and 204 of the 534 CR patients with available tumor samples (Fig. 2A). Overall, a total of 186 non-silent somatic mutations were identified in 121 patients, including 168 missense, 8 insertion or deletion, 6 nonsense, and 4 splice-site mutations, and a preference for C > T/A > G alterations analogous to the somatic single nucleotide

variation (SNV) spectrum in other cancers (Fig. 2B–C and Supplementary Table 1).

As schematically summarized in Fig. 2D, these mutations were involved in BCRs pathway (BCRs related gene, *CARD11*, *LYN*, *CD79A* and *CD79B*), TLRs pathway (TLRs related gene, *MYD88*) and TNFR pathway (TNFR related gene, *TRAF2* and *TNFAIP3*). The most frequent mutations observed were somatic mutations in *MYD88*, which occurred in 50 out of 275 cases (18.2%). The *MYD88* gene encodes an adaptor protein and is composed of an N-terminal Death domain (DD) and a C-terminal Toll-interleukin 1 receptor (TIR) domain (Ngo et al., 2011). All *MYD88* mutations were single nucleotide substitutions, mostly situated in the TIR domain, with the prevalent mutation (an L273P substitution, 37 cases) targeting the conserved B-B loop of the TIR domain. The *CARD11* mutations (33/275, 12.0%) often affected amino acids within or adjacent to the coiled-coil domain of the protein, which were required for BCR-induced NF-κB activation (Lenz et al., 2008). The *LYN* mutations (23/275, 8.4%) were located mostly in PTK, as well as the SH2 domain (Scapini et al., 2009; de Miranda et al., 2014). Mutations of the BCRs proximal adaptors *CD79B/CD79A* occurred in 26 of the 275

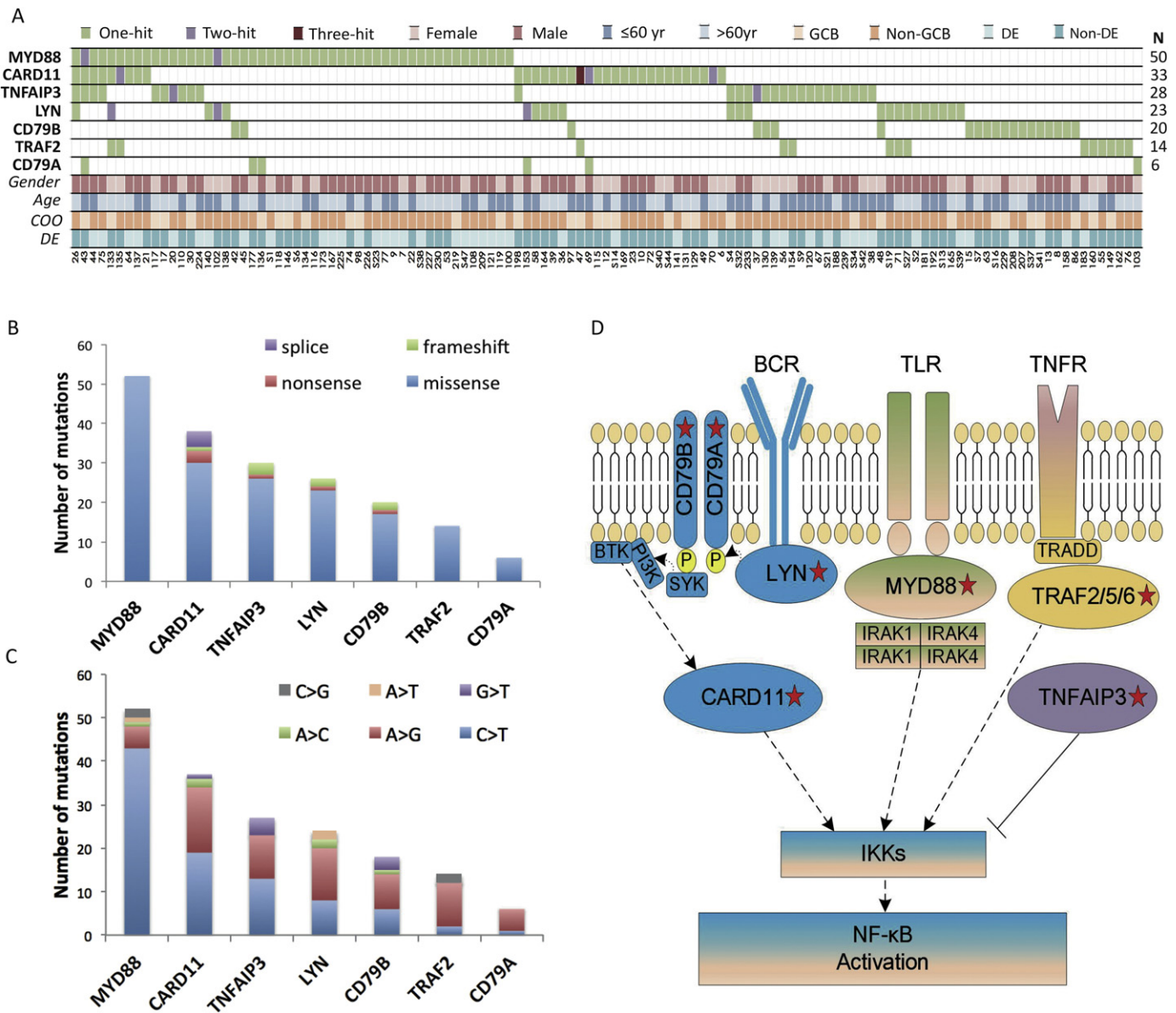


Fig. 2. B-cell function gene mutations in diffuse large B-cell lymphoma (DLBCL). (A) Gene mutations identified by targeted sequencing in 275 patients with DLBCL. (B) Number and type of non-silent somatic mutations. (C) Number and percentage of non-silent somatic SNVs (6 most frequent SNVs were listed on the Figure). (D) Schematic description of B-cell function genes. The key mutated genes are indicated, including *MYD88*, *CARD11*, *TNFAIP3*, *LYN*, *CD79A*, *CD79B* and *TRAF2*. COO: cell of origin; DE: double BCL-2/MYC expression; GCB: germinal center B-cell.

patients (9.5%) and targeted both inside and outside the intracellular immunoreceptor tyrosine-based activation motif (ITAM) (Davis et al., 2010). As for TNFR related gene mutations, *TNFAIP3* (28/275, 10.2%) and *TRAF2* (14/275, 5.1%) mutations were relatively disseminated (Fig. 3A).

The most frequently concurred pairs of genes were *MYD88* and *TNFAIP3* (10 concurrence out of 68 cases, 14.7%), *CARD11* and *LYN* (7 concurrence out of 49 cases, 14.2%), *LYN* and *TRAF2* (4 concurrence out of 33 cases, 12.1%), and *CARD11* and *MYD88* (9 concurrence out of 74 cases, 12.1%, Fig. 3B). Regarding pathological features, 92/175 (52.6%) of the non-GCB patients harbored at least one mutation, significantly higher than those of the GCB patients (29/100, 29.0%, $p < 0.001$). In the non-GCB group, significantly increased proportion of *MYD88* (38/

175 vs 12/100, $p = 0.045$) and *LYN* (20/175 vs 3/100, $p = 0.015$) mutations were observed, as compared to the GCB group (Fig. 3C). As for double BCL-2/MYC expression group, *MYD88* mutations happened more frequently than those without double expression (26.9% vs 13.7%, $p = 0.007$, Fig. 3C).

To determine the role of B-cell function gene mutations on NF- κ B pathway, immunostaining of nuclear NF- κ B1 (p105/p50, classical pathway) and NF- κ B2 (p100/p52, alternative pathway) were performed on 98 patients, including 49 mutated and 49 non-mutated cases with matched clinical and pathological characteristics (Supplementary table 2). Significantly higher fraction of nuclear NF- κ B-positive cells (>30%) was observed in tumors of patients with mutation (34/49, 69.4%) than those without mutation (18/49, 36.7%, $p = 0.002$). The

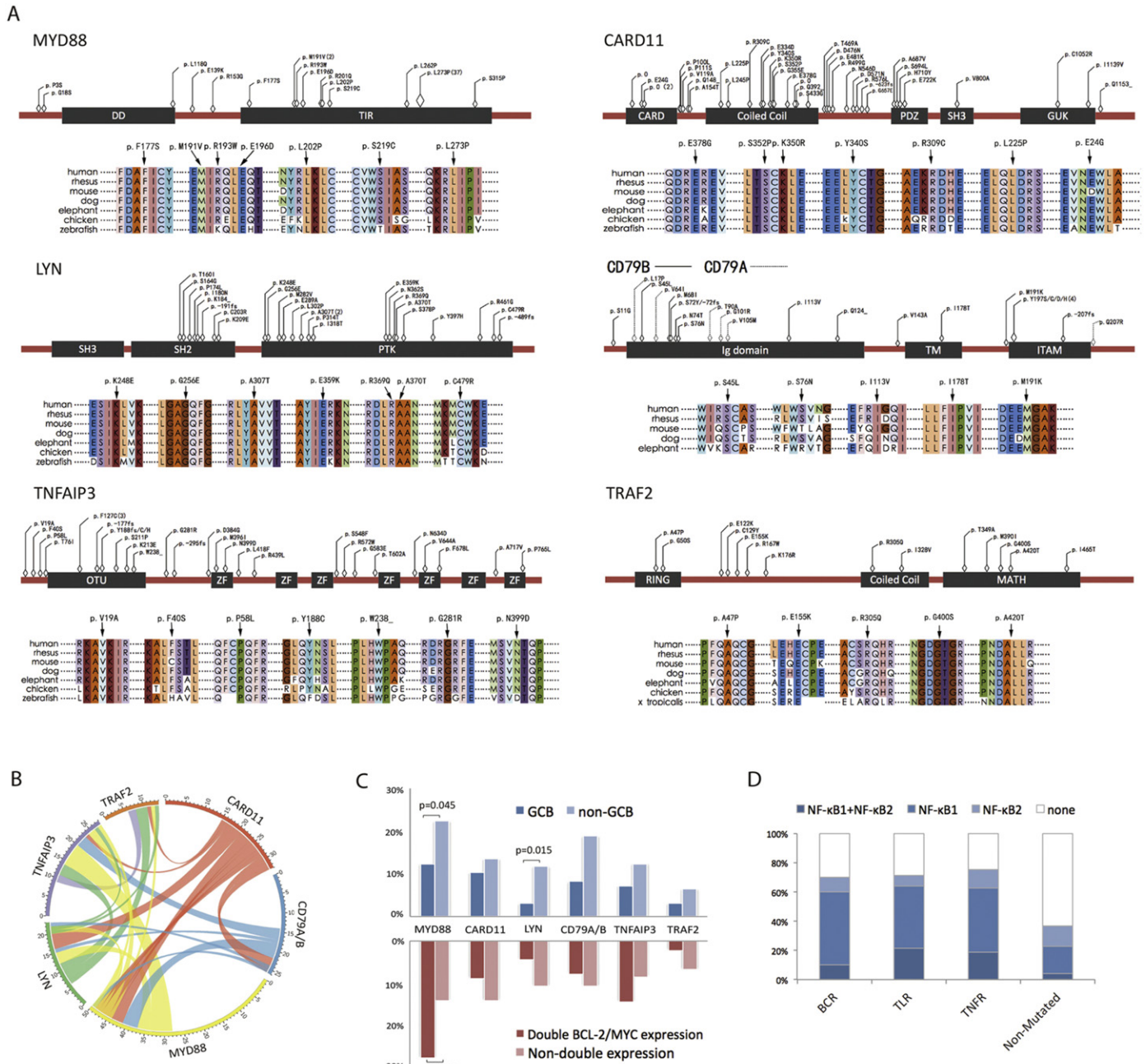


Fig. 3. Mutational patterns of B-cell function genes in diffuse large B-cell lymphoma (DLBCL). A: Schematic location of B-cell function gene mutations, and sequence alignment of protein across distinct species. B: Circos diagram of the correlation between genes, representing the combinations of mutations in different genes. C: Distribution of gene mutations in patients with germinal center B-cell (GCB)/non-GCB subtype and patients with and without double BCL-2/MYC expression. D: Distribution of NF- κ B activation in patients with and without B-cell function gene mutations.

Table 2
Mutational profile of B-cell function genes in the patients with DLBCL.

Mutation	Total (N = 275)	Non-CR (N = 71)	CR (N = 204)		p value ^a	p value ^b
			Additional rituximab (N = 98)	Observation (N = 106)		
BCRs related mutations						
Positive	70/275 (25.5%)	22/71 (31.0%)	26/98 (26.5%)	22/106 (20.8%)	0.409	0.268
Negative	205/275 (74.5%)	49/71 (69.0%)	72/98 (73.5%)	84/106 (79.2%)		
TLRs related mutation						
Positive	50/275 (18.2%)	20/71 (28.2%)	13/98 (13.3%)	17/106 (16.0%)	0.693	0.019
Negative	225/275 (81.8%)	51/71 (71.8%)	85/98 (86.7%)	89/106 (84.0%)		
TNFR related mutations						
Positive	40/275 (14.5%)	16/71 (22.5%)	13/98 (13.3%)	11/106 (10.4%)	0.664	0.032
Negative	235/275 (85.5%)	55/71 (77.5%)	85/98 (86.7%)	95/106 (89.6%)		

^a p value indicated difference between additional rituximab and observation.
^b p value indicated difference between non-CR and CR.

distribution of NF-κB positivity among the three pathways was also similar (Fig. 3D). These results indicated that B-cell function gene mutations are biologically functional and contribute to NF-κB activation.

3.3. Treatment Response

In the univariate analysis, the clinical and pathological factors significantly associated with a lower probability of achieving CR were age > 60 years [odds ratio (OR) = 1.455, 95% confidence interval (CI) 1.182–1.791, p = 0.001], poor performance status (OR = 3.470, 95%CI 2.301–5.233, p < 0.001), advanced Ann Arbor stage (OR = 1.632, 95%CI 1.428–1.866, p < 0.001), elevated serum LDH level (OR = 2.076, 95%CI 1.792–2.406, p < 0.001), multiple extranodal involvement (OR = 2.151, 95%CI 1.598–2.897, p < 0.001) and double BCL-2/MYC

expression (OR = 1.335, 95%CI 1.038–1.718, p = 0.040). In addition, IPI, R-IPI and NCCN-IPI correlated with remission status (p all < 0.001, Table 1). Regarding IPI, the CR rate for low, low-intermediate, intermediate-high and high-risk patient were 91.5%, 77.2%, 58.5% and 47.7%, respectively. Similarly, CR rate for very good, good and poor R-IPI were 94.5%, 83.3% and 53.8%, respectively. CR rate for low, low-intermediate, intermediate-high and high-risk NCCN-IPI were 90.9%, 85.5%, 60.1% and 42.9%, respectively.

Of note, the TLRs and TNFR related gene mutations were more frequently detected in non-CR than in CR patients (OR = 2.275, 95%CI 1.192–4.340, p = 0.019 and OR = 2.182, 95%CI 1.082–4.398, p = 0.032, respectively, Table 2). In the CR group, 17 of 204 patients presented early relapse within 6 months. BCRs, TLRs and TNFR related genes were mutated in 5 (29.4%), 3 (17.6%) and 1 (5.9%) cases, respectively. Although the mutation incidence was higher than the CR group and lower than the non-CR group, no statistical difference was observed, probably due to the limited number of early relapsed patients.

3.4. Survival Analysis

The median follow-up time was 40.5 months (0.6–154.2 months). The 3-year OS of the non-CR patients and the CR patients were 18.4% and 83.3%, respectively.

Among the CR patients, in the univariate analysis, IPI, R-IPI, NCCN-IPI and double BCL-2/MYC expression were significant prognostic factors for both PFS and OS, while COO and BCRs related gene mutations were only for PFS (Table 3). In the multivariate analysis, when R-IPI, IPI, or NCCN-IPI was controlled, double BCL-2/MYC expression and BCRs related gene mutations were independent prognostic factors for PFS (Table 4, Supplementary table 3 and Supplementary table 4). The 3-year PFS rate was 70.9% and 85.8% for patients in remission with and without double BCL-2/MYC expression and 3-year PFS rate was 68.6% and 79.5% for patients positive or negative for BCRs related gene mutations, respectively.

In terms of treatment, the 3-year PFS and OS rate were 79.6% and 89.7% in the AR group, 77.8% and 86.2% in the OBS group, respectively (Fig. 4A). In male patients with low-risk IPI, as well as patients with very good R-IPI and low-risk NCCN-IPI, remarkable improvement of 3-year PFS were observed in the AR group, as compared to those of the OBS group (p = 0.011, p = 0.013, and p = 0.021, respectively), while

Table 3
Univariate analysis of predictors of progression-free survival (PFS) and overall survival (OS) in CR patients with DLBCL.

Variable	PFS			OS				
	HR	95% CI	p value	HR	95% CI	p value		
Gender								
Male vs female	0.893	0.618	1.289	0.545	1.028	0.651	1.623	0.905
International Prognostic Index (IPI)								
Low/low-intermediate/intermediate-high/high	1.930	1.628	2.287	<0.001	2.210	1.790	2.728	<0.001
Revised International Prognostic Index (R-IPI)								
Very good/good/poor	2.467	1.861	3.272	<0.001	3.139	2.179	4.524	<0.001
National Comprehensive Cancer Network (NCCN)-IPI								
Low/low-intermediate/intermediate-high/high	2.081	1.631	2.656	<0.001	2.397	1.784	3.221	<0.001
Cell of origin								
GCB vs non-GCB	0.557	0.346	0.896	0.016	0.701	0.402	1.225	0.212
Double BCL-2/MYC expression								
Positive vs negative	1.971	1.260	3.082	0.003	2.284	1.302	4.007	0.004
Ki-67 > 80%								
Yes vs no	0.875	0.569	1.343	0.541	1.366	0.792	2.356	0.262
BCRs related mutations								
Positive vs negative	2.239	1.251	4.008	0.007	1.651	0.818	3.329	0.162
TLRs related mutations								
Positive vs negative	1.712	0.874	3.352	0.117	1.581	0.688	3.638	0.281
TNFR related mutations								
Positive vs negative	1.337	0.649	2.753	0.431	1.662	0.753	3.669	0.209
Additional Rituximab								
Yes vs no	0.811	0.564	1.166	0.259	0.836	0.533	1.312	0.436

Table 4
Multivariate analysis of predictors of progression-free survival (PFS) and overall survival (OS) in CR patients with DLBCL controlled by Revised International Prognostic Index (R-IPI).

Variable	PFS			OS				
	RR	95% CI	p value	RR	95% CI	p value		
R-IPI								
Very good/good/poor	2.289	1.486	3.526	<0.001	2.723	1.617	4.585	<0.001
Double BCL-2/MYC expression								
Positive vs negative	2.266	1.293	3.973	0.004	2.140	1.098	4.172	0.025
BCRs related mutations								
Positive vs negative	2.192	1.209	3.973	0.010				

3-year OS remained similar (Fig. 4B–D). Moreover, in the subgroup negative for double BCL-2/MYC expression, 3-year PFS was significantly higher in the AR group than in the OBS group ($p = 0.018$, Fig. 4E). According to B-cell function genes, 3-year PFS of the patients with BCRs related gene mutations was also improved in the AR arm ($p = 0.046$, Fig. 4F).

In a Forest plot of univariate analysis on PFS, a favorable response to AR was noted in male patients with low-risk IPI, in patients with very good R-IPI and low-risk NCCN-IPI, subgroup negative for double BCL-2/MYC expression, and with BCRs related gene mutations ($p = 0.015$, $p = 0.021$, $p = 0.030$, $p = 0.022$, and $p = 0.049$, respectively, Fig. 5).

4. Discussion

Prolonged rituximab administration was adopted by several studies in de novo DLBCL patients in the first remission (reviewed in Table 5) (Jaeger et al., 2015; Witzens-Harig et al., 2015; Huang et al., 2012; Habermann et al., 2006). Instead of rituximab maintenance up to three years, we applied rituximab consolidation with two additional doses on patients' intention when CR was achieved. Consistent with the results of rituximab maintenance in the NHL13 trial (Jaeger et al., 2015), the PFS of male DLBCL patients with low-risk IPI was improved by rituximab consolidation in our study. Moreover, irrespective of

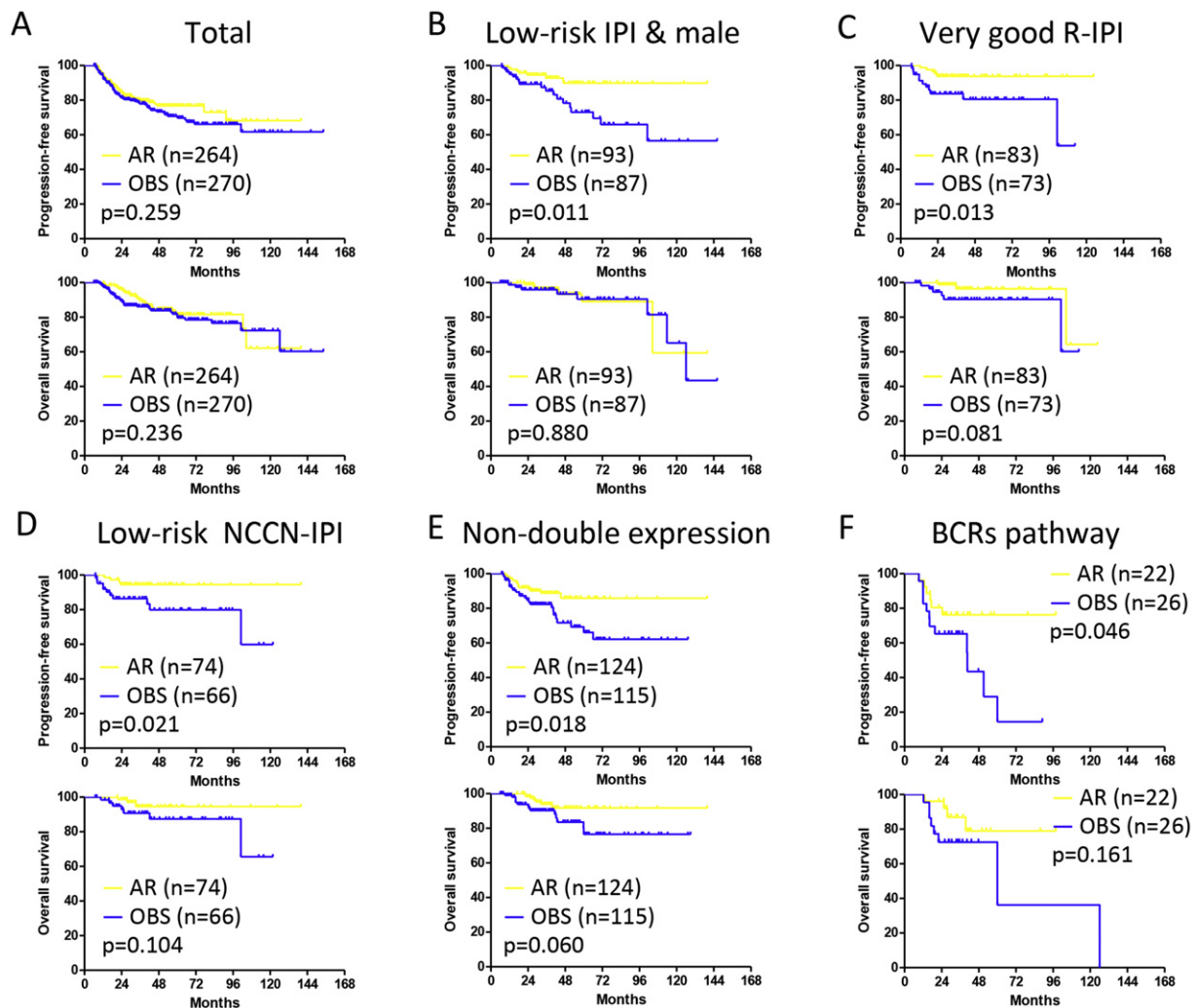


Fig. 4. Progression-free survival and overall survival curves of patients with diffuse large B-cell lymphoma receiving additional rituximab (AR) or observation (OBS). A: Total patients. B: Male patients with low-risk International Prognostic Index (IPI). C: Patients with very good revised IPI (R-IPI). D: Patients with low-risk National Comprehensive Cancer Network (NCCN)-IPI. E: Patients without double BCL-2 and MYC expression. F: Patients with B-cell receptors (BCRs) related gene mutations.

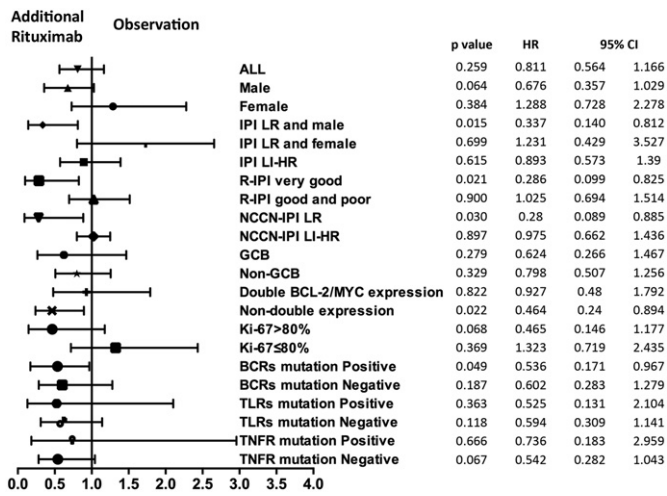


Fig. 5. Forest plot of univariate analysis on progression-free survival of selected subgroups. A shift to the left favored additional rituximab. X-axis: Hazard ratio. IPI: International Prognostic Index; LR: low-risk; HR: high-risk; R-IPI: Revised IPI; NCCN-IPI: National Comprehensive Cancer Network-IPI GCB: germinal center B-cell; BCRs: B-cell receptors; TLRs: Toll-like receptors; TNFR: tumor necrotic factor receptor.

gender, very good R-IPI or low-risk NCCN-IPI patients also achieved the best outcome with rituximab consolidation. Although prospective study is needed for further confirmation, we proposed that low-risk DLBCL may benefit from short-term rituximab consolidation, without long-term rituximab maintenance.

Besides the clinical prognostic indexes, no subgroup analysis of immunophenotypic and genetic characteristics has been performed on response to rituximab. Double BCL-2/MYC expression has recently been recognized as an adverse prognostic factor in DLBCL patients, presenting with 5-year PFS and OS only as 30%, irrespective of CR status (Hu et al., 2013). Our study further revealed that even after CR is achieved, double BCL-2/MYC expression still correlated with poor disease outcome. Interestingly, MYD88 mutation was related to double BCL-2/MYC expression in our study, which could be explained by MYD88-mediated upregulation of BCL-2 (Knittel et al., 2016) and MYC (Rousseau and Martel, 2016). In terms of treatment, the improvement of PFS was observed in DLBCL patients negative for double BCL-2/MYC expression, suggesting another indication for short-term rituximab consolidation. Conversely, prolonged rituximab treatment failed to alter the aggressive clinical course of DLBCL with double BCL-2/MYC expression, which may induce marked stroma- and proliferation-associated genes (Hu et al., 2013) like SPARC (Meyer et al., 2011) and LMO2 (Green et al., 2016) and might confer resistance to rituximab-containing therapy.

Meanwhile, to our knowledge, this is the first report of B-cell function gene mutation profile in a large cohort of Chinese DLBCL patients treated with R-CHOP. Presenting with similar incidence as Western population, B-cell function gene mutations were closely related to DLBCL progression, particularly those involved in TLRs and TNFR pathways, resulting in aberrant activation of NF-κB cascade and resistance to immunochemotherapy in DLBCL (Davis et al., 2010). Clinical trials simply targeting NF-κB have been attempted recent years in patients but most of the results were disappointing (Offner et al., 2015; Leonard et al., 2016). Here we provided evidence that, among mutations involving NF-κB activation, the BCRs pathway, rather than the TLRs and TNFR pathway, was inhibited by rituximab, in consistence with previous basic study (Kheirallah et al., 2010). Thus, the presence of BCR/NF-κB mutations may be more precise in guiding the response to rituximab and referred as a major consideration on rituximab consolidation. As for the TLRs and TNFR related gene mutations, they reflected poor therapeutic response and represented actionable targets for new therapeutic approaches like the BTK inhibitor ibrutinib (Wilson et al., 2015) and IRAK1/4 inhibitor for MYD88 (Li et al., 2015), as well as proteasome inhibitors bortezomib and carfilzomib for TNFAIP3 (Shembade et al., 2010).

In conclusion, the identification of B-cell function gene mutations helped to elucidate molecular heterogeneity in DLBCL. Rituximab consolidation may decrease the risk of relapse in patients with BCRs related gene mutations, as well as those with low-risk and subgroup negative for double BCL-2/MYC expression, providing clues for risk stratification treatment of DLBCL.

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Authorship

P-PX performed the study, collected and analyzed data, and wrote the article. H-JZ performed the experiment and collected clinical data. Y-HH performed the experiment and analyzed the sequencing data. XD-G performed the experiment. XZ collected the tumor samples and related information. YS and SC supervised the clinical data. J-YH

Table 5
Comparison of our study and previous studies on prolonged administration of rituximab in DLBCL.

	Jaeger et al., 2015 Prospective	Witzens-Harig et al., 2015 Prospective	Huang et al., 2012 Retrospective	Habermann et al., 2006 Prospective	Xu et al. Retrospective
Patients	De novo DLBCL and FL3B	B-cell lymphoma, primary and relapsed	De novo DLBCL	De novo DLBCL, 60 years or older	De novo DLBCL
N	683	321	207	415	534
Rituximab	Every 2 months × 12 doses	Every 3 months × 12 doses	Every month × 12 doses	16 doses in 6 months	Every month × 2 doses
End point	EFS	RFS	PFS	FFS	PFS
All	+	+	+	+	+
Male	+	+	+	+	+
Male & IPI ≤ 1	+	+	+	+	+
IPI ≥ 3	NA	NA	NA	NA	NA
CHOP as induction therapy	NA	NA	NA	+	NA
Negative for double BCL-2/MYC expression	NA	NA	NA	NA	+
BCRs related gene mutations	NA	NA	NA	NA	+

NA: not available.

analyzed the sequencing data. S-JC and LW supervised the study. W-LZ designed and supervised the study, and wrote the article.

Disclosures

All authors declare no competing interests.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2017.01.027>.

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