Research Paper

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

Peng-Peng Xu a,1, Hui-Juan Zhong a,1, Yao-Hui Huang a,1, Xiao-Dong Gao a,1, Xia Zhao a,b, Yang Shen a, Shu Cheng a, Jin-Yan Huang a, Sai-Juan Chen a,b, Li Wang a,b, Wei-Li Zhao a,b,*

a State Key Laboratory of Medical Genomics; Shanghai Institute of Hematology; Shanghai Rui Jin Hospital; Shanghai Jiao Tong University School of Medicine; 197 Rui Jin Er Road, Shanghai, China

b Pôle de Recherches Sino-Français en Science du Vivant et Génomique; Laboratory of Molecular Pathology; Shanghai, China

ARTICLE INFO

Article history:
Received 14 January 2017
Accepted 20 January 2017
Available online 21 January 2017

Keywords:
Diffuse large B-cell lymphoma
B-cell function gene mutations
Rituximab
Prognosis

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. 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.

© 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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 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 function gene mutations occurred in 44.0% (121/275) of DLBCL patients. The TFs 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.

http://dx.doi.org/10.1016/j.ebiom.2017.01.027
2352-3964/© 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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 mediatinal 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 μm-paraffin sections with an indirect immunoperoxidase method using antibodies against CD10, BCL-6, MUM-1, Ki-67, BCL-2, MYC, NFκB1 (p105/p50, 1:250, Cell Signaling Technology) and NFκB2 (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% cutoff 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 NFκB localization for >30% of tumor cells was considered positive for NFκB 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 × 48 Access array system (Fluidigm) with FastStart High Fidelity PCR system (Roche) and high-throughput DNA sequencing was performed on illumina Genome Analyzer IIX (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 μl containing 1×GoTaq polymerase (Promega), 0.4 μM of forward and reverse primers, 1.5 mM MgCl2, 200 μM dNTPs and 10 ng DNA.

2.5. Statistical Analysis

Baseline characteristics of patients were analyzed using two-sided χ2 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
Table 1
Clinical and pathological characteristics of the patients with DLBCL.

| Characteristics               | Total (n = 680) | Non-CR (n = 146) | CR (N = 534) | Additional rituximab (n = 264) | Observation (n = 270) | p value<sup>a</sup> | p value<sup>b</sup> |
|-------------------------------|-----------------|------------------|--------------|-------------------------------|-----------------------|----------------------|----------------------|
| Gender                        |                 |                  |              |                               |                       |                      |                      |
| Male                          | 384/680         | 85/146           | 155/264      | 144/270                       | 0.223                 | 0.639                |
| Female                        | 296/680         | 61/146           | 109/264      | 126/270                       |                       |                      |                      |
| Age (years)                   |                 |                  |              |                               |                       |                      |                      |
| ≤60                           | 434/680         | 76/146           | 184/264      | 174/270                       | 0.199                 | 0.001                |
| ≥60                           | 246/680         | 70/146           | 80/264       | 96/270                        |                       |                      |                      |
| Performance status (ECOG)     |                 |                  |              |                               |                       |                      |                      |
| 0–1                           | 604/680         | 109/146          | 246/264      | 249/270                       | 0.741                 | 0.001                |
| ≥2                            | 76/680          | 37/146           | 18/264       | 21/270                        |                       |                      |                      |
| Ann Arbor Stage               |                 |                  |              |                               |                       |                      |                      |
| I–II                          | 330/680         | 38/146           | 141/264      | 151/270                       | 0.554                 | 0.001                |
| III–IV                        | 350/680         | 108/146          | 123/264      | 119/270                       |                       |                      |                      |
| Lactic dehydrogenase          |                 |                  |              |                               |                       |                      |                      |
| Normal                        | 379/680         | 37/146           | 168/264      | 174/270                       | 0.554                 | 0.001                |
| Elevated                      | 301/680         | 109/146          | 96/264       | 96/270                        |                       |                      |                      |
| Extranodal involvement        |                 |                  |              |                               |                       |                      |                      |
| 0–1                           | 545/680         | 96/146           | 219/264      | 230/270                       | 0.554                 | 0.001                |
| >1                            | 135/680         | 50/146           | 45/264       | 40/270                        |                       |                      |                      |
| International Prognostic Index (IPI) |         |                  |              |                               |                       |                      |                      |
| Low                           | 342/680         | 30/146           | 140/264      | 163/270                       | 0.394                 | 0.001                |
| Low-intermediate              | 167/680         | 38/146           | 63/264       | 66/270                        |                       |                      |                      |
| Intermediate-high             | 106/680         | 44/146           | 37/264       | 25/270                        |                       |                      |                      |
| High                          | 65/680          | 34/146           | 15/264       | 16/270                        |                       |                      |                      |
| Revised International Prognostic Index (R-IPI) | |                  |              |                               |                       |                      |                      |
| Very good                     | 165/680         | 9/146            | 83/264       | 73/270                        | 0.109                 | 0.001                |
| Good                          | 342/680         | 57/146           | 129/264      | 156/270                       |                       |                      |                      |
| Poor                          | 173/680         | 80/146           | 52/264       | 41/270                        |                       |                      |                      |
| National Comprehensive Cancer Network (NCCN)-IPI | |                  |              |                               |                       |                      |                      |
| Low                           | 154/680         | 14/146           | 74/264       | 66/270                        | 0.306                 | 0.001                |
| Low-intermediate              | 325/680         | 47/146           | 128/264      | 150/270                       |                       |                      |                      |
| Intermediate-high             | 173/680         | 69/146           | 54/264       | 50/270                        |                       |                      |                      |
| High                          | 28/680          | 16/146           | 8/264        | 4/270                         |                       |                      |                      |
| Cell of origin                |                 |                  |              |                               |                       |                      |                      |
| GCB                           | 192/581         | 36/123           | 77/228       | 79/230                        | 0.992                 | 0.333                |
| non-GCB                       | 389/581         | 87/123           | 151/228      | 151/230                       |                       |                      |                      |
| Double BCL-2/MYC expression   |                 |                  |              |                               |                       |                      |                      |
| Positive                      | 172/470         | 49/108           | 67/191       | 56/171                        | 0.658                 | 0.040                |
| Negative                      | 298/470         | 59/108           | 124/191      | 115/171                       |                       |                      |                      |
| Ki-67 > 80%                   | 263/517         | 58/110           | 113/223      | 92/184                        | 0.921                 | 0.669                |
| No                            | 254/517         | 52/110           | 110/223      | 92/184                        |                       |                      |                      |

<sup>a</sup> p value indicated difference between additional rituximab and observation.
<sup>b</sup> 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...
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-κB pathway, immunostaining of nuclear NF-κB1 (p105/p50, classical pathway) and NF-κB2 (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-κB-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
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 2.192–5.430, p = 0.001) and double BCL-2/MYC expression (OR = 2.135, 95% CI 1.038–4.778, 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 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 both PFS and OS, while COO and BCRs related gene mutations were only for PFS (Table 3).

| Variable                                      | PFS HR | 95% CI | p value | OS HR | 95% CI | p value |
|-----------------------------------------------|--------|--------|---------|-------|--------|---------|
| Gender                                        | 0.893  | 0.618  | 1.289   | 0.545 | 1.028  | 0.651   | 1.623   | 0.905  |
| Male vs female                                |        |        |         |       |        |         |         |       |
| International Prognostic Index (IPI)          | 1.930  | 1.628  | 2.287   | <0.001| 2.210  | 1.790   | 2.728   | <0.001 |
| Low/low-intermediate/intermediate-high/high   |        |        |         |       |        |         |         |       |
| Revised International Prognostic Index (R-IPI)| 2.467  | 1.861  | 3.272   | <0.001| 3.139  | 2.179   | 4.524   | <0.001 |
| Very good/good/poor                          | 2.081  | 1.631  | 2.656   | <0.001| 2.397  | 1.784   | 3.221   | <0.001 |
| National Comprehensive Cancer Network (NCCN)-IPI|        |        |         |       |        |         |         |       |
| Cell of origin                                |        |        |         |       |        |         |         |       |
| Intermediate-high/high                        |        |        |         |       |        |         |         |       |
| 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%                                   | 2.329  | 1.251  | 4.008   | 0.007 | 1.651  | 0.818   | 3.292   | 0.162  |
| Yes vs no                                     | 1.712  | 0.874  | 3.352   | 0.117 | 1.581  | 0.688   | 3.638   | 0.281  |
| BCRs related mutations                        |        |        |         |       |        |         |         |       |
| Positive vs negative                          | 1.337  | 0.649  | 2.753   | 0.431 | 1.662  | 0.753   | 3.669   | 0.209  |
| Additional Rituximab                          | 0.811  | 0.564  | 1.166   | 0.259 | 0.836  | 0.533   | 1.312   | 0.436  |

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

| Mutation                      | Total (N = 275) | Non-CR (N = 71) | Additional rituximab (N = 98) | Observation (N = 106) | p valuea | p valueb |
|-------------------------------|-----------------|-----------------|-------------------------------|-----------------------|----------|----------|
| BCRs related mutations        |                 |                 |                               |                       |          |          |
| Positive                      | 70/275          | 22/71           | 26/98                         | 22/106                | 0.409    | 0.268    |
| Negative                      | 205/275         | 49/71           | 72/98                         | 84/106                |          |          |
| TLRs related mutation         |                 |                 |                               |                       |          |          |
| Positive                      | 50/275          | 20/71           | 13/98                         | 17/106                | 0.693    | 0.019    |
| Negative                      | 225/275         | 51/71           | 85/98                         | 89/106                |          |          |
| TNFR related mutations        |                 |                 |                               |                       |          |          |
| Positive                      | 40/275          | 16/71           | 13/98                         | 11/106                | 0.664    | 0.032    |
| Negative                      | 235/275         | 55/71           | 85/98                         | 95/106                |          |          |

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

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

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

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).

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

Table 5 is needed for further analysis. 

Table 5

| Additional Rituximab | Observation | p value | HR | 95% CI |
|----------------------|------------|---------|----|--------|
| ALL                  |            | 0.158   | 0.831| 0.544| 1.296 |
| Male                 |            | 0.064   | 0.676| 0.357| 1.029 |
| Female               |            | 0.184   | 1.288| 0.728| 2.278 |
| IPI LR and male      |            | 0.655   | 0.357| 0.140| 0.812 |
| IPI LR and female    |            | 0.699   | 1.231| 0.429| 3.527 |
| R-IPI very good       |            | 0.021   | 0.986| 0.949| 0.985 |
| R-IPI good and poor   |            | 0.900   | 1.025| 0.694| 1.514 |
| NCCN-IPI LR          |            | 0.030   | 0.28  | 0.089| 0.885 |
| NCCN-IPI LHR         |            | 0.897   | 0.875| 0.546| 1.436 |
| TLRs                 |            | 0.179   | 0.524| 0.266| 1.467 |
| Non-TLRs             |            | 0.325   | 0.790| 0.567| 1.256 |
| Double BCL-2/MYC positive |       | 0.021   | 0.986| 0.949| 0.985 |
| Double BCL-2/MYC negative |       | 0.432   | 1.059| 0.526| 2.091 |
| Ki-67 > 80%          |            | 0.068   | 0.465| 0.346| 1.377 |
| Ki-67 ≤ 50%          |            | 0.389   | 1.382| 0.739| 2.529 |
| BCRs mutation Positive|          | 0.049   | 0.536| 0.171| 0.967 |
| BCRs mutation Negative|         | 0.187   | 0.602| 0.283| 1.279 |
| TLNs mutation Positive|         | 0.363   | 0.525| 0.313| 2.104 |
| TLNs mutation Negative|         | 0.118   | 0.954| 0.309| 3.141 |
| TNFR mutation Positive|        | 0.464   | 0.736| 0.183| 2.959 |
| TNFR mutation Negative|        | 0.667   | 0.542| 0.292| 1.033 |

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.

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.

Table 5

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

| Patients | 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 |
|----------|--------------------------------|--------------------------------------|--------------------------------|----------------------------------|------------------------|
| N        | De novo DLBCL and FLJ6 Every 2 months × 12 doses | De novo DLBCL 207 Every month × 12 doses | De novo DLBCL, 60 years or older 415 16 doses in 6 months | De novo DLBCL 534 Every month × 2 doses |
| Rituximab | 683 Every 2 months × 12 doses | 321 Every 3 months × 12 doses | | |
| End point | EFS | PFS | RFS | OS | PFS | OS | PFS | OS |
| All      | − | + | + | (in DLBCL) | − | NA | NA | NA | − | − |
| Male     | − | + | + | (in DLBCL) | − | NA | NA | NA | − | − |
| IPI ≥ 3  | NA | NA | NA | NA | NA | + | NA | NA | + | − |
| CHOP as induction therapy | NA | NA | NA | NA | NA | + | NA | NA | + | − |
| Negative for double BCL-2/MYC expression | NA | NA | NA | NA | NA | NA | NA | NA | + | − |

NA: not available.

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 recently 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 TNAFAP3 (Shembade et al., 2010).

Funding

This study was supported, in part, by research funding from the National Natural Science Foundation of China (81325003, 81520108003, 81670716 and 81201863), the Shanghai Commission of Science and Technology (14430723400, 14140903100 and 16JC1405800), Shanghai Municipal Education Commission Gaofeng Clinical Medicine Grant Support (20152206 and 20152208), Multi-center clinical research project by Shanghai Jiao Tong University School of Medicine (DLY201601), SMC-Chen Xing Scholars Program, Chang Jiang Scholars Program, Collaborative Innovation Center of Systems Biomedicine and the Samuel Waxman Cancer Research Foundation.

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 study, collected and analyzed data, and wrote the article. J-YH related information. YS and SC supervised the clinical data. J-YH collected the tumor samples and collected clinical data. H-JZ performed the experiment and analyzed the sequencing data. YS and SC supervised the clinical data. J-YH

P-P. Xu et al. / EBioMedicine 16 (2017) 106–114
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.

References

Alizadeh, A.A., Eisen, M.B., Davis, R.E., et al., 2000. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 403, 503–511.

Cheson, B.D., Horning, S.J., Coiffrer, B., et al., 1999. Report of an international workshop to standardize response criteria for non-Hodgkin’s lymphomas. NCI Sponsored International Working Group. J. Clin. Oncol. 17, 1244.

Cheson, B.D., Pfistner, B., Junweid, M.E., et al., 2007. Revised response criteria for malignant lymphoma. J. Clin. Oncol. 25, 579–586.

Compagno, M., Lim, W.K., Grunn, A., et al., 2009. Mutations of multiple genes cause deregulation of NF-kappab in diffuse large B-cell lymphoma. Nature 459, 717–721.

Davis, R.E., Ngo, V.N., Lenz, G., et al., 2010. Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature 463, 88–92.

de Miranda, N.F., Georgiou, K., Chen, L., et al., 2014. Exome sequencing reveals novel mutation targets in diffuse large B-cell lymphomas derived from Chinese patients. Blood 124, 2544–2553.

Green, T.M., Jensen, A.K., Holst, R., et al., 2016. Multiplex polymerase chain reaction-based prognostic models in diffuse large B-cell lymphoma patients treated with R-CHOP. Br. J. Haematol. 174, 876–886.

Green, T.M., Young, K.H., Visco, C., et al., 2012. Immunohistochemical double-hit score is a strong predictor of outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J. Clin. Oncol. 30, 3460–3467.

Habermann, T.M., Weller, E.A., Morrison, V.A., et al., 2006. Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma. J. Clin. Oncol. 24, 3121–3127.

Hans, C.P., Weisenburger, D.D., Greiner, T.C., et al., 2004. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 103, 275–282.

Honma, K., Tsuzuki, S., Nakagawa, M., et al., 2009. TNAFIP3/A20 functions as a novel tumor suppressor gene in several subtypes of non-Hodgkin lymphomas. Blood 114, 2467–2475.

Horn, H., Ziepert, M., Becher, C., et al., 2013. MYC status in concert with BCL2 and BCL6 expression predicts outcome in diffuse large B-cell lymphoma. Blood 121, 2253–2263.

Hu, S., Xu-Monette, Z.Y., Tzankov, A., et al., 2013. MYC/BCL2 protein coexpression contributes to the inferior survival of activated B-cell subtype of diffuse large B-cell lymphoma and demonstrates high-risk gene expression signatures: a report from The International DLBCL Rituximab-CHOP Consortium Program. Blood 121, 4021–4031 (quiz 4250).

Huang, B.T., Zeng, Q.C., Yu, J., et al., 2012. How to determine post-RCHOP therapy for risk-tailored adult patients with diffuse large B-cell lymphoma, addition of maintenance rituximab or observation: multicenter experience. J. Cancer Res. Clin. Oncol. 138, 125–132.

IPR. 1993. A predictive model for aggressive non-Hodgkin’s lymphoma. The International Non-Hodgkin’s Lymphoma Prognostic Factors Project. N. Engl. J. Med. 329, 987–994.

Jaeger, U., Trneny, M., Melzer, H., et al., 2015. Rituximab maintenance for patients with aggressive B-cell lymphoma in first remission: results of the randomized NHL13 trial. Haematologica 100, 955–963.

Johnson, N.A., Slack, G.W., Savage, K.J., et al., 2012. Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J. Clin. Oncol. 30, 3452–3459.

Kheirallah, S., Caron, P., Gross, E., et al., 2010. Rituximab inhibits B-cell receptor signaling. Blood 115, 985–994.

Knittel, G., Liedergens, P., Korovkina, D., et al., 2016. B-cell-specific conditional expression of Myd88p.L252P leads to the development of diffuse large B-cell lymphoma in mice. Blood 127, 2732–2741.

Lenz, G., Davis, R.E., Ngo, V.N., et al., 2008. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. Science 319, 1676–1679.

Leonard, J.P., Kollababa, K., Reeves, J.A., et al., 2016. Randomized phase 2 open-label study of R-CHOP+/- bortezomib in patients (Pts) with untreated non-germinal center B-cell-like (non-GCB) subtype diffuse large cell lymphoma (DLBCL): results from the pyramid trial (NCT00931918). [ASH abstract 811]. Blood 126 (suppl 23).

Li, Z., Younger, K., Gartenhaus, R., et al., 2015. Inhibition of IRAK1/4 sensitizes T cell acute lymphoblastic leukemia to chemotherapies. J. Clin. Invest. 125, 1081–1097.

Meyer, P.N., Fu, K., Greiner, T., et al., 2011. The stromal cell marker SPARC predicts for survival in patients with diffuse large B-cell lymphoma treated with rituximab. Am. J. Clin. Pathol. 135, 54–61.

Ngo, V.N., Young, R.M., Schmitz, R., et al., 2011. Oncogenically active MYD88 mutations in human lymphoma. Nature 470, 115–119.

Offner, F., Samoilova, O., Osmanov, E., et al., 2015. Frontline rituximab, cyclophosphamide, doxorubicin, and prednisone with bortezomib (VR-CAP) or vincristine (R-CHOP) for non-GCB DLBCL. Blood 126, 1893–1901.

Rousseau, S., Martel, C., 2016. Gain-of-function mutations in the toll-like receptor pathway; TPL2-mediated ERK1/ERK2 MAPK activation, a path to tumorigenesis in lymphoid neoplasms? Front. Cell Dev. Biol. 4, 50.

Rossi, D., Ciardullo, C., Gaidano, C., 2013. Genetic aberrations of signaling pathways in lymphomagenesis: revelations from next generation sequencing studies. Semin. Cancer Biol. 23, 422–430.

Scapini, P., Pereira, S., Zhang, H., et al., 2009. Multiple roles of Lyn kinase in myeloid cell signaling and function. Immunol. Rev. 228, 23–40.

Sehn, L.H., Berry, B., Chhanabhai, M., et al., 2007. The revised International Prognostic Index (R-IPI) is a better predictor of outcome than the standard IPI for patients with diffuse large B-cell lymphoma treated with R-CHOP. Blood 109, 1857–1863.

Shembade, N., Ma, A., Harjai, E.W., 2010. Inhibition of NF-kappab signaling by A20 through disruption of ubiquitin enzyme complexes. Science 327, 1135–1139.

WHO classification of tumors of haematopoietic and lymphoid tissues. In: Sh, S. (Ed.), International Agency for Research on Cancer, fourth ed. World Health Organization classification of tumors, Lyon.

Wilson, W.H., Young, R.M., Schmitz, R., et al., 2015. Targeting B cell receptor signaling with ibrutinib in diffuse large B cell lymphoma. Nat. Med. 21, 922–926.

Witzens-Harig, M., Benner, A., Mcclanahan, F., et al., 2015. Rituximab maintenance improves survival in male patients with diffuse large B-cell lymphoma. Results of the HD2002 prospective multicentre randomized phase III trial. Br. J. Haematol. 171, 716–719.

Zhou, Z., Sehn, L.H., Rademaker, A.W., et al., 2014. An enhanced International Prognostic Index (NCCN-IPI) for patients with diffuse large B-cell lymphoma treated in the rituximab era. Blood 123, 837–842.