A Genetic Predictive Model for Precision Treatment of Diffuse Large B-Cell Lymphoma with Early Progression

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Abstract

Early progression after standard immunochemotherapy leads to a very dismal outcome and necessitates alternative treatment for patients with Diffuse Large B-Cell Lymphoma (DLBCL). This study aimed to develop a genetic predictive model for early progression and evaluate its potential in advancing alternative treatment. Thirty-two hotspot driver genes were examined in 145 DLBCL patients and 5DLBCL cell lines using next-generation sequencing. By analyzing the association of clinical features, cell-of-origin, double expression, positive p53 protein, and gene alterations with early progression, a powerful genetic predictive model incorporating lactate dehydrogenase levels, CD79B mutations, and PIM1 mutations was developed which was validated in the other cohort. The potential of novel treatment based on the modeling was investigated in in vitro DLBCL cell lines and in vivo xenograft mouse models. CD79B and PIM1 mutations were significantly associated with double expression and indicated a better response to inhibitors of BTK (ibrutinib) and pan-PIM kinase (AZD 1208) through repressing activated oncogenic signaling. Since the two inhibitors failed to decrease BCL2 level, BCL2 inhibitor (venetoclax) was added and demonstrated to enhance their apoptosis-inducing activity in mutant cells with double expression. The genetic predictive model provides a robust tool to identify early progression and determine alternative treatment.

Keywords: Diffuse large B-cell lymphoma; Early progression; CD79B; PIM1

Introduction

Diffuse Large B-Cell Lymphoma (DLBCL) is the most common subtype of non-Hodgkin’s lymphoma with great heterogeneity in genetics, manifestations, therapy responses, and prognoses. The first-line standard immunochemotherapy has achieved a complete cure or yielded a long-term survival in over 60% of patients. However, the rest eventually succumbed to recurrent or refractory disease [1,2]. Particularly, those with early progression within less than 12 months (POD12) usually experienced a very dismal outcome and did not benefit much from salvage therapy in combination with autologous stem cell transplantation [3]. Hence, it is necessary to provide the man alternative treatment beyond standard immunochemotherapy in the setting of frontline therapy. Firstly, it is important to introduce a powerful predictive model for POD12 in newly diagnosed DLBCL patients. Currently, International Prognostic Index (IPI) based on five clinical parameters and Cell of Origin (COO) classification into Germinal-Center B-cell-like (GCB), Activated B-Cell-like (ABC), and unclassified type 3 subtypes have been used for prognostic assessment in DLBCL [4,5]. Concurrent BCL2 and c-MYC and/or BCL6 rearrangements, Double Expression (DE) of c-MYC and BCL2, and positive p53 protein expression have also been reported to be associated with poor outcomes [6-9]. The subset involving c-MYC and BCL2 and/or BCL6 rearrangements has been classified into high-grade double-hit or triple-hit B-cell lymphoma, for which intensive-dose regimens are recommended in the frontline therapy [8,10]. However, accurate prediction of other prognostic models for POD12 remains elusive. In addition, optimal alternative treatment beyond standard immunochemotherapy has not been determined. It has been reported that the addition of novel targeted agents, such as Bortezomib, Lenalidomide, and Ibrutinib did not meet the primary endpoint of improving overall survival in clinical trials designed based on COO [11,12].
Recently, many gene alterations have been identified using whole-exome and transcriptome sequencing in DLBCL samples [13-15]. Some of these alterations have been confirmed to drive tumor development and promote DLBCL cell proliferation and survival via regulating oncogenic signaling pathways. In this context, several genetic prognostic models incorporating gene alterations have been established to predict the outcomes, and outperformed the currently known models such as IPI, COO, and DE [14]. These prognostication tools have not only extended our understanding of DLBCL pathogenic mechanisms, but also uncovered potential opportunities for precision treatment strategies. For example, CD79B mutations involved in the B-Cell Receptor (BCR) signaling result in BCR-dependent activation of NF-κB [16]. Some new molecular subtypes are characteristic of CD79B mutations, such as MCD termed by both CD79B and MYD88L265P mutations [15], and cluster 5, which is a unique genetic signature of ABC DLBCL and enriched with CD79B mutations [13]. Ibrutinib, a BTK inhibitor, has been observed to induce better response in DLBCL patients with CD79B mutations [15,17,18]. Increasing evidence suggests that targeted agents should be evaluated in DLBCL clinical trials in the context of subtype-specific genetic aberrations and activating mutations that positively modulate oncogenic signaling pathways.

Herein, we analyzed a panel of 32 genes with high frequency of mutations in DLBCL, which have been reported to contribute to tumorigenesis and progression [15,19]. A robust genetic predictive model for POD12 was established after evaluating the association of traditional prognostic factors and gene alterations with POD12. This genetic predictive model suggests a novel treatment strategy by targeting specific gene alterations, which was successfully confirmed using in vitro DLBCL cell lines and in vivo xenograft mouse models.

Materials and Methods

Patients and cell lines

One hundred and forty-five patients with newly diagnosed DLBCL were enrolled in this study. The diagnosis of DLBCL was confirmed by at least two pathologists in accordance to the World Health Organization classification [20], and patients with double-hit and triple-hit were excluded from the study. All patients were treated with standard immunochemotherapy in the frontline setting. A cohort of 84 DLBCL patients was used for validation. Patient characteristics are shown in Supplementary Table 1. This study was reviewed and approved by the hospital Institutional Review Boards with informed consent of the patients.

Five human DLBCL cell lines were used in the study, including OCI-Ly8, Ros50, OCI-Ly3, OCI-Ly7, and Val. They have been authenticated and monitored for Mycoplasma contamination.

Next-generation sequencing

Genomic DNA was extracted from formalin-fixed paraffin-embedded tumor tissues of DLBCL patients using a QIAamp DNA FFPE Tissue kit (Qiagen) or from cultured cells using the TIANamp genomic DNA kit (Tiangen). High-throughput DNA sequencing was performed on Illumina Genome Analyzer Miseq (Illumina) according to the manufacturer’s instructions. Quality of DNA libraries was assessed using a bioanalyzer high sensitivity DNA chip (Agilent Technologies). VarDict (v1.4.6) [21] and Varscan (v2.4.2) [22] were utilized to call Single Nucleotide Polymorphism (SNP) and small indel from the BAM files. The variants were filtered including the aligned reads depth of variant over 500-fold with frequency of over 2%, and the allele frequency of lower than 5% in the 1000G, ESP or ExAC database.

Immunohistochemistry

Immunohistochemistry was performed on 3-μm paraffin sections with an indirect immunoperoxidase method using antibodies against CD10 (Abcam, 1:500), BCL6 (Abcam, 1:500), MUM1 (Abcam, 1:250), BCL2 (Abcam, 1:250), c-MYC (Abcam, 1:250), and p53 (Abcam, 1:50). COO subgroups were determined using the Han’s classification [23]. DE of BCL2 and c-MYC was defined as cut-off value of 50% and 40%, respectively. The cut-off value of 50% was considered as p53 protein positive.

Cell proliferation

Cells in log growth phase were inoculated into 96-well plates in triplicate at a density of 2 × 10^6/ml. After treatment with different concentrations of drugs, 20 μl of CCK-8 reagent (Meilunbio) was added, and continued to culture for another 4 h. Cell viability was quantified by reading absorbance at 450 nm on an automatic microplate reader (Thermo Fisher 1510 Vantaa, Finland).

Cell apoptosis

After treatment with different drugs, 5 × 10^5 cells were washed and resuspended in 100 μl of 1 × binding buffer containing 5 μl Annexin-V (BD Pharmingen) and 5 μl 7-AAD (BD Pharmingen). Following incubation for another 15 min at room temperature in the dark, cell suspension was added with 400 μl of 1 × binding buffer, and then analyzed on a FACScan. The lower right-hand and the upper right-hand quadrant cells were considered apoptotic.

Western blot

Total protein was extracted with RIPA buffer (Beyotime), and nuclear and cytoplasmic fractions were isolated using the nuclear and cytoplasmic protein extraction kit (Beyotime). Western blot was carried out following the standard protocol with the following primary antibodies, β-actin (Trans, 1:5,000), BCL2 (Abcam, 1:1,500), c-MYC (Abcam, 1:1,500), phosphorylated-CDC25A (Abcam, 1:500), H3 (Abcam, 1:1,000), MCL1 (CST, 1:1,000), BCL-XL (CST, 1:1,000), p65 (CST, 1:1,000), IκB-α (CST, 1:1,000). Protein bands were visualized using the enhanced chemiluminescence system (Beyotime) according to the manufacturer’s instruction.

Xenograft mouse model

Six-week-old SCID mice (Charles River) were subcutaneously injected with OCI-Ly8 and Val cells in the posterior flank. When tumor sizes approached 150 mm^3, mice were randomly divided into control and AZD1208 groups. AZD1208 (50 μg/g) wasted daily after being formulated in 0.5% CMC-Na solution. Tumor size was measured every other day and estimated by applying the following formula: (3.14 × length × width^2)/6. Animals were maintained and manipulated in accordance with the principles of laboratory animal care under the Institutional Animal Care and Use Committee-approved protocol.

Statistical analysis

Clinical features, molecular biomarkers, and genes mutations were compared using t-test for continuous variables and χ^2-test for categorical variables. Predictive model was assessed using the Area under Receiver Operating Characteristic (AUROC). Progression-Free Survival (PFS) was calculated from the date of initial diagnosis to the time of recurrence, death or the last follow-up. Overall Survival (OS) was measured from the date of initial diagnosis to the death...
or the last follow-up. PFS and OS were estimated using the Kaplan-Meier method and the log-rank test was used for comparison between groups. Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) 21.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as p<0.05.

**Results**

**CD79B and PIM1 mutations are independently related to POD12 following first-line immunochemotherapy**

Total 32 hotspot driver genes were examined using next-generation sequencing in 145 newly diagnosed patients with DLBCL. All of these genes were mutated in 91.72% patients (133/145) with a median number of 4 (0-24), including single nucleotide variants, frame shift mutations, insertions, and deletions. SNPs were filtered according to the defined criteria. The frequency of CD79B (42.86% vs. 9.38%, p=0.000) and PIM1 mutations (38.78% vs. 17.71%, p=0.005) showed a significant increase in patients with POD12 (n=49) (Figure 1), but no difference was found in median number of mutations (5 vs. 4, p=0.287). When the associations of gender, age, ECOG score, Ann Arbor stage, LDH level, the number of extranodal involvement, IPI score, COO, DE, and positive p53 protein with POD12 were evaluated, univariate analysis displayed an obvious correlation with Ann Arbor stage (p=0.019), LDH level (p=0.001), IPI score (p=0.014), and DE (p=0.001) (Table 1). Multivariate analysis, including Ann Arbor stage, LDH level, DE, and gene mutations of CD79B and PIM1, revealed that LDH level (OR=2.990, p=0.018), CD79B (OR=5.970, p=0.001), and PIM1 mutations (OR=3.021, p=0.026) were independently correlated with POD12.

**CD79B and PIM1 mutations connect with complex genetic events and unfavorable features and survival**

Complex genetic events were seen involving in CD79B and PIM1 mutations (Figure 2A). There were 36 mutational sites occurring in 30 patients with CD79B mutations, including 8 in the Ig-like V type domain, 5 in the transmembrane domain, and 23 in the Immunoreceptor Tyrosine-based Activation Motif (ITAM). A prominent site was identified at the Y196 of ITAM in 63.3% (19/30) of patients. Moreover, 25 gene alterations were observed to accompany with CD79B mutations, only PIM1 (14/36, p=0.002) and MYD88 L265P (11/16, p=0.000) mutations having significant correlation. 105 mutational sites of PIM1 were seen in 36 patients, a majority of which occurred in the kinase domain with V177 (4/36, 11.1%), S188 (6/36, 16.7%) and E226 (6/36, 16.7%) having high frequency involvement. There were 30 gene alterations accompanying with PIM1 aberrations, including IRF4 (9/14, p=0.001) and MYD88L265P (10/16, p=0.000) alterations having obvious correlation.

By analyzing clinicopathological features, CD79B mutations were significantly associated with DE and non-GCB subtype, and PIM1 mutations were statistically relevant to DE and advance stage (Supplementary Table 2). Patients with CD79B mutations manifested poorer PFS and OS than wild-type patients, while patients with PIM1 mutations presented poorer PFS, but not OS (Figure 2B). In a larger cohort [14], both CD79B- and PIM1-mutantpatients were found to have worse survival than those with wild-type genes (Figure 2C). These data indicate that CD79B and PIM1 mutations are associated with complex genetic events and unfavorable prognosis.

**Incorporating the variables CD79B mutations, PIM1 mutations, and LDH levels create a robust predictive model for POD12**

We established a new genetic predictive model for POD12 after integrating LDH levels (OR=2.990, p=0.018), CD79B mutations (OR=5.970, p=0.001), and PIM1 mutations (OR=3.021, p=0.026), which were independently related to POD12. In this genetic predictive model, LDH levels and PIM1 mutations were defined as a score of 1, and CD79B mutations was assigned as a score of 2 based on their OR value. The analysis of AUROC (0.771, 95% CI: 0.689-

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<th>Table 1: Clinical characteristics of patients grouped by POD12.</th>
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<td><strong>Characteristics</strong></td>
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<td>Male, n (%)</td>
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<td><strong>IPI factors</strong></td>
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<td>Age &gt;60 years, n (%)</td>
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<td>LDH level&gt;normal, n (%)</td>
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<td>ECOG=1, n (%)</td>
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<td>Extraneal involvement &gt;1 site, n (%)</td>
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<td><strong>IPI score</strong></td>
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<td>Intermediate-high/high risk (3-5), n (%)</td>
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<td><strong>Co-expression MYC and BCL2</strong></td>
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<td>Yes, n (%)</td>
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<td><strong>COO</strong></td>
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<td>Non-GCB, n (%)</td>
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0.853) demonstrated the model to have a good performance. With the highest Youden’s index of 0.4052, scores of 2-4 were recommended to distinguish low- and high-risk patients of POD12 with a sensitivity of 55.10% and a specificity of 85.42%. The incidence of POD12 was significantly increased in patients with scores of 2-4 compared with those with scoring 0-1 (21.15% vs. 65.85%, p=0.000), who also displayed poorer PFS and OS (Figure 3A, 3B). The genetic predictive model was successfully validated in a cohort of 84 cases and in another larger cohort of 1001 patients [14] (Figure 3C, 3D). Our modeling was further compared with traditional IPI score and new molecular subtypes, MCD and cluster 5, on the power for predicting POD12. The result showed that this model outperformed conventional IPI score and MCD subtype (Figure 3E). The association between cluster 5 and POD12 was not found (12/64, p=0.576) by analyzing reported data [13]. Collectedly, we created a predictive model for POD12 with a powerful performance by incorporating the variables CD79B mutations, PIM1 mutations, and LDH levels.

CD79B and PIM1 mutations indicate better response to BTK and pan-PIM kinase inhibitors, and BCL2 inhibitor enhances their apoptosis-inducing effects in mutant cells with DE

A novel treatment was evaluated based on our predictive model. By sequencing a panel of 32 hotspot driver genes in 5 DLBCL cell lines, only Val cells had a CD79B mutation in the ITAM domain (T212M), and OCI-Ly8 cells had PIM1 mutations in the kinase domain (S188N and L284F), which were both accompanied by mutations in MYC, BCL2, FOXO1, and CREBBP. Val and OCI-Ly8 cell lines were used to test the effects of CD79B and PIM1 mutations on DLBCL cells sensitivity to BTK inhibitor Ibrutinib and pan-PIM kinase inhibitor AZD 1208. Cell proliferation assay was carried out to determine

![Figure 1: The diagram of the frequencies of hotspot gene mutations in POD12 and non-POD12 patients.](image1)

![Figure 2: The sites of CD79B and PIM1 mutations and comparison of survival between mutant and wild-type patients. (A) Complex genetic events were involved in the CD79B and PIM1 mutations. (B) PIM1-mutant (n=36, p=0.004) and CD79B-mutant (n=30, p=0.000) patients had poorer PFS than wild-type patients. CD79B-mutant patients displayed poorer OS (n=30, p=0.001), while PIM1-mutant patients had an indifferent OS (n=36, p=0.862). (C) PIM1-mutant (n=166, p=0.002) and CD79B-mutant (n=47, p=0.028) patients were observed to have worse OS in a larger DLBCL cohort.](image2)
Figure 3: A new genetic predictive model for POD12 including CD79B mutation, PIM1 mutation, and LDH levels. (A) In this genetic predictive model, a significant difference in the incidence of POD12 was found among patients with scores of 0-1 (n=104) and 2-4 (n=41) (21.15% vs. 65.85%, p=0.000). (B) The patients with score of 0-1 (n=104) and 2-4 (n=41) had significantly different PFS (p=0.000) and OS (p=0.018) with inverse correlation. (C) This genetic predictive model was validated in a cohort of 84 DLBCL cases. (D) The association of scores of 2-4 (n=119) with poorer survival was confirmed in a larger cohort (p=0.000). (E) This genetic predictive model for POD12 outperformed the IPI score and MCD subtype.

Figure 4: Correlation of CD79B and PIM1 mutations with better response to BTK and pan-PIM inhibitors. (A) Ibrutinib and AZD 1208 showed a dose- and time-dependent inhibition of cell growth. (B) CD79B-mutated Val cells and PIM1-mutated OCI-Ly8 cells were more susceptible to Ibrutinib- and AZD 1208-induced proliferation inhibition when compared with wide-type cells (p<0.01). (C) and (D) CD79B-mutated Val cells and PIM1-mutated OCI-Ly8 cells were more sensitive to Ibrutinib- and AZD 1208-induced apoptosis when compared with wide-type cells (p<0.01).
experimental doses of Ibrutinib and AZD1208 (Figure 4A). We found that CD79B-mutant Val cells were more susceptible to 10 µM Ibrutinib-induced growth inhibition and apoptosis when compared with CD79B-wildtype OCI-Ly8 cells (p<0.01); PIM1-mutant OCI-Ly8 cells also presented a better response to 40 µM AZD 1208 than PIM1-wildtype Val cells (p<0.01) (Figures 4B-4D). The significance of PIM1 mutations was further confirmed in xenograft mouse models. After AZD 1208 was given daily according to the protocol, tumor growth was significantly suppressed in OCI-Ly8 xenograft mice when compared with Val xenografts (Figure 5A). These results suggest that CD79B and PIM1 mutations make DLBCL cells sensitive to BTK and pan-PIM kinase inhibitors.

Next, we determined the action mechanisms of the two inhibitors by Western blot. A gradual increase of cytoplasmic IκB and a reduction
of nuclear p65 were found after Ibrutinib treatment for 24 h to 72 h, while phosphorylated-CDC25A was decreased following AZD 1208 treatment for 24 h to 72 h (Figure 5B). Both c-MYC and BCL2 proteins were found to present in Val and OCI-Ly8 cells. However, c-MYC expression, but not BCL2 expression, was significantly reduced by Ibrutinib and AZD1208 (Figure 5C). Therefore, BCL2 inhibitor venetoclax was added to enhance the effectiveness of Ibrutinib and AZD 1208. We picked 0.1 µM venetoclax for experiment based on the results of cell proliferation assay (Supplementary Figure 1). Encouragingly, venetoclax did show a prominent synergistic effect when combined with Ibrutinib and AZD 1208, even though it alone did not produce obvious apoptosis (Figure 6A). The observed effect of venetoclax should be attributed to its inhibition of BCL2 function, since BCL2 levels were not affected (Figure 6B). The combinatorial effect of other key agents for DLBCL therapy, including rituximab, doxorubicin, and lenalidomide, were also examined with Ibrutinib and AZD 1208. Their doses in in vitro assay were also chosen based on the results of cell proliferation assay (Supplementary Figure 1). The results showed that 100 µg/ml rituximab, 15 ng/ml doxorubicin, and 50 µM lenalidomide only produced rather limited synergistic action of apoptosis with Ibrutinib and AZD 1208 (Figure 6A). Although some of them exerted inhibitory effects on BCL-XL and MCL1, they have no effect on BCL2 expression (Figure 6B), suggesting the importance of blocking BCL2 in promoting apoptosis of mutant cell with DE.

**Discussion**

We observed a panel of 32 high-frequency mutated genes and a median number of 4 mutations in DLBCL patients, which are similar to the findings in the study containing larger amount of Candidate Cancer Genes (CCGs) [13]. To make up for the deficiency of lacking patient-matched normal samples, we developed a computational method to filter germline variants and artifacts. Even though it did not completely exclude rare germline variants, some evidence has indicated that these rare germline variants have minimal effects on the detection of CCGs [13].

By excluding patients with double-hit and triple-hit from this study, we successfully identified a new subset of DLBCL patients prone to early progression and in need of frontline alternative treatment with the use of a novel genetic predictive model. Simultaneously, we demonstrated that our genetic predictive model for POD12 has the advantage over conventional prognostic models such as IPI, COO, DE, positive p53 protein, and new prognostic models like MCD subtype and Cluster 5 genetic signature. MCD subtype has inferior outcome following standard immunochemotherapy [14,15]. Most of patients are ascribed to ABC DLBCL and have a tendency of extensive extranodal involvement [24]. Chapuy et al. [13] integrated recurrent mutations, somatic copy number alterations, and structural variants to recognize a unique ABC-type cluster 5, which exhibited frequent mutations in CD79B and MYD88 L265P and was associated with extranodal tropism and inferior survival. PIM1 mutations have been addressed to be frequent in patients with MCD subtype and cluster 5. However, their associations with POD12 have not been reported before.

Complex genetic events were observed in the CD79B and PIM1 mutations, including their connectivity with MYD88 L265P and IRF4. The prominent mutation at Y196 of the CD79B ITAM domain has been demonstrated to enhance the BCR signaling [16]. Although the consequences of other mutations are not clear, current evidence supports their influence on the activation of the BCR signaling [25]. PIM1 has been linked to the initiation and progression of malignant phenotype by regulating cell cycle progression and inhibiting apoptosis. Many mutational sites of PIM1 were observed with predominant mutations at V177, S188 and E226 of the kinase domain. PIM1 alterations have been demonstrated to affect the structural stability and kinase activity of PIM1 [26,27]. It was confirmed about unfavorable features of advanced stage, non-GCB, and DE and poor survival in CD79B- and PIM1-mutant patients.

In agreement with previous study [28], we demonstrated that CD79B and PIM1 mutations signal better response to BTK and pan-PIM kinase inhibitors by suppressing oncogenic signaling. However, these two inhibitors induced limited apoptosis in CD79B- or PIM1-mutant cells with DE. Later mechanistic studies revealed that they decreased the expression of c-MYC, but had no impacts on BCL2, who persistent expression largely reduced the drug potency. Indeed, DLBCL patients responding to Ibrutinib often experience a rapid PD in clinical practice [17]. A strong synergy of venetoclax with BTK and pan-PIM kinase inhibitors was found in these DE cells, although it alone did not induce obvious apoptosis. Some studies have reported that venetoclax alone rarely exhibits a notable efficacy in other B-cell malignancies except chronic lymphocytic leukemia [29,30]. We also found that other key agents for DLBCL therapy, such as rituximab, doxorubicin, and lenalidomide, produced less synergistic activity with BTK and PIM1 inhibitors. Even though some of them reduced the expression of BCL-XL and MCL1, they did not significantly affect the BCL2 expression. These findings suggest a key role of blocking BCL2 in promoting apoptosis of DLBCL cells with DE.

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**References**