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Loss of \textit{PRDM1}/BLIMP-1 function contributes to poor prognosis of activated B-cell-like diffuse large B-cell lymphoma

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoid malignancy, accounting for 30–40% of all non-Hodgkin lymphoma.$^{1}$ Anthracycline-based combination chemotherapy, first introduced in the 1970s, is the backbone of therapy for patients with DLBCL, and currently the standard therapy regimen includes the anti-CD20 antibody rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone.$^{2-4}$ Despite encouraging complete remission and overall survival (OS) rates using this regimen, up to one-third of DLBCL patients suffer from relapse or refractory primary disease.$^{5}$

The heterogeneous clinical outcome of DLBCL results in part from variable genetic profiles of this tumor. Gene expression profiling (GEP) has identified two distinct types of DLBCL: germinal center B-cell-like and activated B-cell-like (GCB and ABC). Patients with ABC-DLBCL have markedly poorer survival than do patients with GCB-DLBCL.$^{6,7}$ However, the molecular mechanism responsible for this difference is not completely understood.

ABC-DLBCL is characterized by constitutive nuclear factor-kB (NF-kB) activation and genetic alterations that interfere with terminal B-cell differentiation.$^{8}$ \textit{PRDM1}, located on chromosome 6q21, encodes for BLIMP-1, a zinc-finger-containing DNA-binding transcriptional repressor. BLIMP-1 expression is required for the development of immunoglobulin-secreting cells and maintenance of long-lived plasma cells.$^{9}$ Conditional \textit{PRDM1}-knockout mice do not have production of plasma cells or serum immunoglobulins.$^{10}$ \textit{PRDM1} is frequently inactivated in ABC-DLBCL cases as a result of genetic deletions or mutations or transcriptional repression of it.$^{11,12}$ Studies have demonstrated that an inactivation mutation of \textit{PRDM1} is recurrent in ~25% of ABC-DLBCL cases.$^{11,14}$ Moreover, conditional deletion of \textit{PRDM1} in murine B cells facilitates the development of lymphoproliferative disease resembling human ABC-DLBCL.$^{6,11}$

Limited research has been carried out on the clinical implications of \textit{PRDM1}/BLIMP-1 abnormality in DLBCL patients. In the present study, we investigated \textit{PRDM1} deletion and mutation and
BLIMP-1 protein expression in a large cohort of de novo DLBCL patients treated with rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone. Our results demonstrated that disruption of PRDM1/BLIMP-1 is associated with poor prognosis for ABC-DLBCL. We further characterized the potential molecular mechanisms underlying the tumor-suppressive function of PRDM1/BLIMP-1 using GEP.

MATERIALS AND METHODS

Patients

We studied 520 biopsy specimens obtained from rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone-treated patients with de novo DLBCL. The diagnostic criteria for DLBCL, patient-selection process, therapy and treatment responses were described previously. The study was approved by the institutional review boards of the participating institutions.

Immunohistochemistry

Tissue microarrays prepared using formalin-fixed, paraffin-embedded tissue blocks of the diagnostic biopsy specimens obtained from the studied patients were stained with an anti-BLIMP-1 antibody (EPR16655; Epitomics, Burlingame, CA, USA). BLIMP-1 expression levels were determined by estimating the percentage of BLIMP-1+ tumor cells in the tissue sections. Evaluation of other biomarkers was performed using immunohistochemistry with corresponding antibodies. Details of the immunohistochemical procedures and scoring processes were described previously.

Fluorescence in situ hybridization, and PRDM1 and TP53 sequencing

Fluorescence in situ hybridization (FISH) probes spanning the PRDM1 gene and chromosome 6 centromere were generated as described previously. Dual-color FISH was performed using standard procedures. Briefly, 4-μm-thick tissue sections were baked overnight at 74 °C and incubated for 24 h at 37 °C in a hybridization oven (ThermoBrite, Buffalo Grove, IL, USA). Posthybridization washes were performed in 0.4× standard saline citrate (0.3% NP-40) for 2 min and 2× standard saline citrate (0.1% NP-40) for 1 min at 72 °C. Slides were then mounted with 4,6-diamidino-2-phenylindole (0.5 g/ml) containing an antifade solution. For one slide containing 25 cases, 200 nuclei of cells were evaluated independently by two observers (AT and VM). The overall concordance of their evaluations was nearly perfect (~0.95), thus all other slides (200 nuclei per case) were scored by AT only. The ratio of PRDM1 signals (red) to CEP6 signals (green) was calculated. If this ratio was lower than 0.72, heterozygous PRDM1 deletion was considered to be present. Ratios lower than 0.38 were considered to be suggestive of homozygous PRDM1 deletions. These ratios were calculated as ratios below the mean plus three standard deviations of green to red signal ratios in reference cases (tonsils; n = 6) and subtraction of tumor-infiltrating T cells, which accounted for ~30% of undeleted alleles. MYC, BCL2 and BCL6 gene arrangements and copy-number aberrations were detected using FISH, as well.

Sequencing of PRDM1 coding regions was performed by Polymorphic DNA Technologies (Alameda, CA, USA) using Sanger sequencing. Sequencing results were compared with the NCBI NM_001198 reference sequence, followed by exclusion of all the single-nucleotide polymorphisms documented by the NCBI dbSNP database (build 147). The remaining variants detected by sequencing were considered as PRDM1 somatic mutations. TP53 was sequenced using a p53 AmpliChip (Roche Molecular Diagnostics, Pleasanton, CA, USA) as described previously.

GEP and GCB/ABC classification

GEP was performed using a GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) with total RNAs as described previously. The CEL files were deposited in the National Center for Biotechnology Information Gene Expression Omnibus repository (GSE no. 31312). GEP classified 407 cases as GCB/ABC subtypes, and the cell of origin of another 111 cases was determined and compared by immunohistochemical algorithms (Hans, Visco-Young and Chou). Cell-of-origin classification based on B-cell-associated gene signatures has been described previously.

Microarray profiling

An HTG EdgeSeq Whole Transcriptome Assay (HTG Molecular Diagnostics, Tucson, AZ, USA) coupled with a HiSeq system (Illumina, San Diego, CA, USA) was used for measuring the expression of microRNAs (miRNAs) in formalin-fixed, paraffin-embedded tissues (unpublished data). Selection of regulatory miRNAs for BLIMP-1 expression was based on TargetScan and published data.

Statistical analysis

Clinical and molecular features of patient tumors were compared using the Fisher’s exact or χ2 test. OS and progression-free survival (PFS) in the study population were analyzed using the Kaplan–Meier method, and differences between DLBCL subgroups were compared using the log-rank test with the Prism 6 software program (GraphPad Software, San Diego, CA, USA). Multivariate analysis was performed using the Cox proportional hazards regression model with the SPSS software program (version 19.0; IBM Corporation, Armonk, NY, USA). P-values of up to 0.05 were considered statistically significant.

RESULTS

Patients

The clinical characteristics of the study patients with DLBCL (n = 520) are summarized in Table 1. Their median age was 65 years (range, 16–82 years), the median follow-up duration was 46.7 months (range, 20.0–186.7 months) and the male:female ratio was 1.2:1. Two hundred sixty-eight patients (52%) had GCB-DLBCL, and 250 patients (48%) had ABC-DLBCL.

Homozygous PRDM1 deletion predicts poor prognosis for ABC-DLBCL

We detected homozygous deletion of PRDM1 in 19 (7%) of 292 patients with available FISH results (Figure 1a and Supplementary Table 1). Patients with homozygous PRDM1 deletions had lower BLIMP-1 protein expression than did those without these deletions (P = 0.055; Figure 1a). Homozygous PRDM1 deletion was not associated with the ABC phenotype (P = 0.34) or any other clinical parameters. OS was significantly shorter in patients with homozygous PRDM1 deletions than in those with normal or heterozygous deletions of PRDM1 or monosomy 6 (P = 0.037) (Figure 1b). This difference was remarkable in patients with ABC-DLBCL (P = 0.004) but not in those with GCB-DLBCL (P = 0.98) (Figure 1b). Homozygous PRDM1 deletion was also associated with significantly poorer PFS in the entire DLBCL cohort (P = 0.0048) and in ABC-DLBCL (P = 0.036) (Figure 1c). Multivariate analysis adjusting clinical features confirmed homozygous PRDM1 deletion as an independent prognostic factor for both OS (P = 0.032) and PFS (P = 0.037). In the ABC-DLBCL patients, homozygous PRDM1 deletion was an independent prognostic factor for OS (P = 0.032) but not for PFS (P = 0.13) (Table 2).

PRDM1 mutation predicts poor prognosis for ABC-DLBCL

We studied PRDM1 mutations in 368 patients with available genomic DNA. Using a quality score cutoff of 16 (97% confidence), we identified that 94 patients (25.5%) had PRDM1 mutations within the coding DNA sequence region of PRDM1 (Supplementary Table 2). Of these, seven patients had truncating (nonsense) mutations and two had a frameshift mutation. Seven of these nine patients had ABC-DLBCL, including four patients...
with short OS and PFS (Supplementary Table 3). PRDM1 mutation was not associated with the ABC phenotype \((P = 0.91)\) and did not impact survival in the overall cohort or in GCB-DLBCL patients in particular (Supplementary Figure 1). However, PRDM1 mutation did correlate with poorer prognosis for ABC-DLBCL (Figure 2a). Moreover, ABC-DLBCL patients harboring PRDM1 mutations within exon 1 or 2 had markedly shorter survival durations than did patients with mutations in exons 3–7 or wild-type PRDM1 (Figures 2b and c). In comparison, the prognostic impact of exon 1 and 2 PRDM1 mutations on GCB-DLBCL was minimal (Supplementary Figure 2). Consistently, we found that patients with exon 1 and 2 PRDM1 mutations were more likely to have overexpression of both Myc and Bcl-2 than were patients with exon 3–7 mutations in the overall cohort \((P = 0.03)\) and those with wild-type PRDM1 in ABC-DLBCL \((P = 0.05)\) but not in those with GCB-DLBCL \((P = 0.44)\). Multivariate analysis of clinical parameters, Myc/Bcl-2 expression scores, and PRDM1 mutations indicated that Myc/Bcl-2 coexpression (hazard ratio: 2.9, \(P < 0.001\)), but not PRDM1 mutations (hazard ratio: 1.54, \(P = 0.12\)), was an independent prognostic factor in ABC-DLBCL.

### Table 1. Clinical features of DLBCL patients with BLIMP-1 expression, PRDM1 mutation and homozygous deletion

| Variables                        | BLIMP-1 protein expression | PRDM1 mutation | Homozygous PRDM1 deletion |
|----------------------------------|----------------------------|----------------|---------------------------|
|                                  | Positive | Negative | P-value | Positive | Negative | P-value | Positive | Negative | P-value |
| No. of patients                  | 132      | 388      |         | 94      | 274      |         | 19       | 273      |         |
| Cell-of-origin                   |          |          |         |          |          |         |          |          |         |
| GCB                              | 39 (30%) | 229 (59%)| <0.0001 | 48 (51%) | 141 (52%)| 0.91    | 7 (37%)  | 138 (51%)| 0.34    |
| ABC                              | 92 (70%) | 158 (41%)|          | 46 (49%) | 131 (48%)|          | 12 (63%) | 134 (49%)|          |
| Plasmablastic subtype            |          |          |         |          |          |         |          |          |         |
| No                               | 55 (47%) | 259 (94%)| <0.0001 | 25 (80%) | 48 (87%) | 0.41    | 9 (100%) | 185 (87%)| 0.27    |
| Yes                              | 29 (53%) | 15 (6%)  |          | 6 (20%)  | 7 (13%)  |          | 0        | 25 (13%) |          |
| Age (years)                      |          |          |         |          |          |         |          |          |         |
| < 60                             | 53 (40%) | 166 (43%)| 0.61    | 39 (41%) | 117 (43%)| 0.90    | 10 (53%) | 114 (42%)| 0.47    |
| ≥ 60                             | 79 (60%) | 222 (57%)|          | 55 (59%) | 157 (57%)|          | 9 (47%)  | 159 (58%)|          |
| Gender                           |          |          |         |          |          |         |          |          |         |
| Male                             | 81 (61%) | 224 (58%)| 0.46    | 51 (54%) | 162 (59%)| 0.47    | 11 (58%) | 143 (52%)| 0.81    |
| Female                           | 51 (39%) | 164 (42%)|          | 43 (46%) | 112 (41%)|          | 8 (42%)  | 130 (48%)|          |
| Stage                            |          |          |         |          |          |         |          |          |         |
| I and II                         | 60 (47%) | 179 (47%)| 0.96    | 36 (41%) | 120 (45%)| 0.54    | 11 (58%) | 126 (48%)| 0.48    |
| III and IV                       | 67 (53%) | 198 (53%)|          | 52 (59%) | 147 (55%)|          | 8 (42%)  | 135 (52%)|          |
| B symptoms                       |          |          |         |          |          |         |          |          |         |
| No                               | 78 (61%) | 244 (67%)| 0.24    | 48 (55%) | 176 (68%)| 0.039   | 15 (79%) | 165 (63%)| 0.22    |
| Yes                              | 50 (39%) | 122 (33%)|          | 39 (45%) | 84 (32%) |          | 4 (21%)  | 98 (37%) |          |
| LDH level                        |          |          |         |          |          |         |          |          |         |
| Normal                           | 49 (41%) | 134 (38%)| 0.58    | 30 (34%) | 107 (43%)| 0.17    | 8 (44%)  | 102 (40%)| 0.80    |
| Elevated                         | 72 (59%) | 222 (62%)|          | 57 (66%) | 140 (57%)|          | 10 (56%) | 156 (60%)|          |
| No. of extranodal sites          |          |          |         |          |          |         |          |          |         |
| 0–1                              | 93 (74%) | 287 (77%)| 0.51    | 65 (72%) | 200 (77%)| 0.39    | 17 (94%) | 197 (78%)| 0.13    |
| ≥ 2                              | 33 (26%) | 87 (23%) |          | 25 (28%) | 59 (23%) |          | 1 (6%)   | 57 (22%) |          |
| ECOG performance status          |          |          |         |          |          |         |          |          |         |
| 0–1                              | 99 (84%) | 289 (83%)| 0.83    | 61 (72%) | 208 (86%)| 0.005   | 16 (89%) | 204 (83%)| 0.75    |
| ≥ 2                              | 19 (16%) | 59 (17%) |          | 24 (28%) | 34 (14%) |          | 2 (11%)  | 43 (17%) |          |
| Size of largest tumor (cm)       |          |          |         |          |          |         |          |          |         |
| < 5                              | 61 (64%) | 162 (56%)| 0.19    | 38 (61%) | 129 (59%)| 0.88    | 10 (67%) | 129 (54%)| 0.43    |
| ≥ 5                              | 35 (36%) | 128 (44%)|          | 24 (39%) | 88 (41%) |          | 5 (33%)  | 108 (46%)|          |
| IPI score                        |          |          |         |          |          |         |          |          |         |
| 0–2                              | 77 (59%) | 231 (62%)| 0.59    | 50 (54%) | 166 (63%)| 0.14    | 13 (68%) | 160 (61%)| 0.51    |
| 3–5                              | 53 (41%) | 142 (38%)|          | 42 (46%) | 96 (37%) |          | 6 (32%)  | 103 (39%)|          |
| Therapy response                 |          |          |         |          |          |         |          |          |         |
| CR                               | 103 (78%)| 286 (74%)| 0.32    | 65 (69%) | 206 (75%)| 0.28    | 14 (74%) | 214 (78%)| 0.58    |
| PR                               | 17 (13%) | 52 (13%) |          | 18 (19%) | 35 (13%) |          | 4 (21%)  | 29 (11%) |          |
| SD                               | 6 (4.5%) | 18 (5%)  |          | 4 (4%)   | 12 (4%)  |          | 1 (5%)   | 11 (4%)  |          |
| PD                               | 6 (4.5%) | 32 (8%)  |          | 7 (7%)   | 21 (8%)  |          | 0        | 19 (7%)  |          |

Abbreviations: ABC, activated B-cell-like; CR, complete response; DLBCL, diffuse large B-cell lymphoma; ECOG, Eastern Cooperative Oncology Group; GCB, germinal center B-cell-like; IPI, International Prognostic Index; LDH, lactate dehydrogenase; PD, progressive disease; PR, partial response; SD, stable disease. Bold values are the significant \(P\) values or those showing trends of significance.
We also studied the correlations between various types of PRDM1 mutations and BLIMP-1 protein expression, as well as PRDM1 deletion. Patients with nonsense mutations or exon 1/2 mutations had lower BLIMP-1 expression than patients without these aberrations, but the P-values for the differences were not significant (Supplementary Figure 3). No significant associations were observed between PRDM1 mutations and PRDM1 allelic deletions.

Table 2. Multivariate analysis for homozygous PRDM1 deletions in DLBCL and ABC-DLBCL

|                      | Overall survival | Progression-free survival | Overall survival | Progression-free survival |
|----------------------|------------------|---------------------------|------------------|---------------------------|
|                      | P-value | HR     | 95% CI | P-value | HR     | 95% CI | P-value | HR     | 95% CI | P-value | HR     | 95% CI |
| Female sex           | 0.60     | 0.89   | 0.58–1.37 | 0.69    | 0.92   | 0.62–1.37 | 0.63    | 1.15   | 0.66–1.99 | 0.71    | 1.10   | 0.67–1.82 |
| B symptoms           | 0.031    | 1.62   | 1.04–2.50 | 0.072    | 1.45   | 0.97–2.19 | 0.26    | 1.37   | 0.79–2.37 | 0.21    | 1.39   | 0.83–2.32 |
| IPI > 2              | < 0.001  | 3.02   | 1.96–4.64 | < 0.001  | 2.62   | 1.76–3.89 | < 0.001  | 2.67   | 1.57–4.53 | < 0.001  | 2.29   | 1.40–3.75 |
| Tumor size > 5 cm    | 0.18     | 1.33   | 0.87–2.02 | 0.56     | 1.12   | 0.64–1.66 | 0.26    | 1.35   | 0.80–2.26 | 0.60    | 1.14   | 0.70–1.84 |
| PRDM1 homozygous deletion | 0.032  | 2.23   | 1.07–4.65 | **0.037** | 2.09   | 1.05–4.18 | **0.032** | 2.58   | 1.08–6.15 | **0.13** | 1.95   | 0.83–4.59 |

Abbreviations: ABC, activated B-cell like; HR, hazard ratio; CI, confidence interval; DLBCL, diffuse large B-cell lymphoma; IPI, International Prognostic Index. Bold values are the significant P values or those showing trends of significance.

Figure 1. Homozygous PRDM1 deletion in DLBCL cases. (a) Representative examples of FISH results with heterozygous PRDM1 deletion (of note are a mixture of cells with two green signals, corresponding to centromere 6, but lacking red signals, corresponding to PRDM1, and cells with two green signals and one red signal), homozygous PRDM1 deletion (of note are two green signals but a lack of red signals in the majority of the cells; one cell in the center of the microphotograph has both red and green signals and serves as an internal positive control) and monosomy 6 (all cells have only one green and one red signal). DLBCL patients with homozygous PRDM1 deletions had lower levels of BLIMP-1 protein expression than did the rest of the studied patients. (b and c) The impact of homozygous PRDM1 deletion on OS and PFS in all patients with DLBCL, patients with ABC-DLBCL and patients with GCB-DLBCL. Patients with this deletion had shorter OS and PFS durations than did patients with normal FISH signals, heterozygous PRDM1 deletions or monosomy 6. This trend was greater in patients with ABC-DLBCL than in GCB-DLBCL.
Loss of BLIMP-1 protein expression correlates with Myc overexpression and decreased p53 pathway molecule expression in ABC-DLBCLs.

Representative immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections for BLIMP-1 protein expression is shown in Figure 3a. Using a cutoff of 10%, we found that 132 (25%) of the 520 patients were positive for BLIMP-1. BLIMP-1 protein expression was more common in patients with the ABC phenotype (37% vs 15% in GCB-DLBCL, \( P < 0.0001 \)) and plasma-blast subtype of DLBCL than others \( (P < 0.0001; \text{Table 1}). \) The histogram of BLIMP-1 expression in the study cohort, and distribution of PRDM1 deletions and mutations and BLIMP-1 protein expression in 180 patients with all data available is shown in Figure 3b. Ninety-six of these patients were in the ABC subgroup, 59 of whom (61%) had BLIMP-1− DLBCL. Thirty-seven (63%) of the BLIMP-1− ABC-DLBCL cases had no detectable PRDM1 deletions or mutations. MiRNA profiling identified that miR-30d-3p, miR-30d-5p and miR-30b-5p were significantly upregulated in BLIMP-1− patients without apparent PRDM1 genetic aberrations, suggesting that epigenetic regulations may also have a role in loss of PRDM1/BLIMP-1 expression.

In the ABC subgroup, BLIMP-1 negativity was associated with reduced protein expression for the NF-κB pathway component p65 \( (P = 0.028) \), p53 \( (P = 0.024; \text{only in subjects wild type for } P53) \), p53 downstream targets MDM2 \( (P = 0.002) \) and p21 \( (P = 0.074) \), and phosphorylated AKT \( (P = 0.0095) \). BLIMP-1 negativity also was more common in patients without expression of CD30 \( (P = 0.0082) \) (Table 3). Of note, Myc expression was increased in BLIMP-1− cases, with a trend of significance \( (P = 0.085) \), and this negative correlation between BLIMP-1 and Myc overexpression in ABC-DLBCL was statistically significant when using a higher cutoff \( (\geq 20\%) \) for BLIMP-1+ \( (P = 0.0062) \).

In the GCB subgroup, BLIMP-1 negativity was associated with MYC translocation \( (P = 0.049) \), mutated p53 overexpression, and decreased p21 expression \( (P = 0.0028) \) (Table 3). We found several other alterations associated with BLIMP-1 negativity in the overall DLBCL cohort, including BCL2 translocation \( (P = 0.004) \), TP53 mutation \( (P = 0.005) \), decreased expression of p63 \( (P = 0.023) \), nuclear p50 \( (P = 0.023) \) and IRF4/MUM1 \( (P < 0.0001) \), increased expression of GCET1 \( (P = 0.039) \) and CD10 \( (P = 0.019) \), and a lack of BCL6 translocation. Bcl-6 expression tended to be upregulated in BLIMP-1− DLBCLs \( (P = 0.092) \), especially in patients with mutated TP53 \( (P = 0.075) \).

We did not observe correlations between BLIMP-1 expression and survival in the study cohort (Supplementary Figure 4). However, BLIMP-1 negativity had an unfavorable impact on OS and PFS in DLBCL patients with TP53 mutations, although the impact was not significant \( (OS, P = 0.12; PFS, P = 0.074) \) (Figure 3c).
Homozygous $PRDM1$ deletion contributes to gene expression signatures of transcriptional activation.

We identified 25 differentially expressed genes (DEGs) between homozygous $PRDM1$ deletion-positive and -negative DLBCL cases (Figure 4a). Expression of all of the DEGs was upregulated. Subjects with homozygous $PRDM1$ deletions had robust transcriptional activity with upregulation of transcription factors $STAT3$, $HLF$, $TCF19$, $TSHZ1$, and $HMGA2$. Accordingly, analysis of the ABC...
subgroup also identified increased transcriptional signatures (Table 4). In contrast, we found no DEGs for homozygous PRDM1 deletion in the GCB subgroup.

PRDM1 expression signatures in the overall cohort resemble those in ABC-DLBCL
We compared GEP of BLIMP-1− and BLIMP-1+ DLBCL cases in the overall DLBCL cohort, in wild-type and mutated PRDM1 subsets (Figure 4a). DEGs were identified in the overall DLBCL and the subset with wild-type PRDM1 (147 and 32 DEGs, respectively) with a false discovery rate (FDR) threshold of 0.01, but not in the subset with mutated PRDM1, suggesting loss of wild-type BLIMP-1 function (Table 4). Loss of BLIMP-1 expression in DLBCL was associated with downregulation of PRDM1, X-box binding protein 1 (XBP1), which encodes for a critical regulator of plasma differentiation, and its downstream target genes involved in endoplasmic reticulum, protein synthesis and transcription.

Moreover, expression of genes related to immunoglobulin production (ELL2, MGC29506/MZB1 and ARID3A), cell differentiation (BATF, IRF4, which transactivates PRDM1 and represses BLCL expression), and B/T cell receptor signaling inhibition (LAX1) was also downregulated in BLIMP-1− DLBCLs. In contrast, expression of CD22, MS4A1, which encodes for CD20, BCL11A (a B-cell proto-oncogene and cofactor with Bcl-6, upregulated during hematopoietic cell differentiation) and BLK, which is involved in B-cell receptor (BCR) signaling, was markedly upregulated (1.42–1.54, 1.67 and 1.2-fold, respectively).

DEGs were also identified in the ABC and GCB subgroups (38 DEGs in ABC with an FDR threshold of 0.05 (Table 4); 22 DEGs in GCB with an FDR threshold of 0.15; Supplementary Table 4). When analyzed in the wild-type and mutated PRDM1 subsets separately, DEGs were only identified in ABC-DLBCL (but not GCB-DLBCL) with wild-type PRDM1 (30 DEGs with a FDR threshold of 0.30; Figure 4a and Table 4). The spectrum of DEGs in the ABC subgroup was similar to that in the overall cohort, including downregulation of PRDM1, XBP1, GHTM, DUSP4, TMEM59, APOBEC3B, FKB111, DNAJC3, DNAJ5, MANF, SEC24A, VDR, TXND5C, ITGAL and C10orf55, as well as upregulation of BCL11A, CD22 and CLU1 in BLIMP-1− ABC-DLBCL cases. IG1 was downregulated in BLIMP-1− ABC-DLBCL, whereas CD37 antigen was upregulated. In comparison, the DEGs identified in the overall GCB-DLBCL (regardless of PRDM1 mutation status) also included PRDM1, BCL11A and DUSP4, but were more likely to be involved in immune responses (CTLA4, GTP5, C10orf58, HLA-F and SERPINC1) and metabolism (ACSL1, LCAT2, GTPBP8, CTSA and METRNL) (Supplementary Figure 5a).

Considering the potential prognostic impact of BLIMP-1 expression in DLBCL patients with mutated TP53, we additionally compared the BLIMP-1 expression signatures in patients with mutated TP53 and in those wild type for TP53. Forty-three and 33 DEGs were identified, respectively, in these two subsets with an FDR threshold of 0.20 (Supplementary Figures 5b–c and Supplementary Table 4). Comparing these two BLIMP-1 GEP signatures, we found that PRDM1, SRR1 and HSP90B1 were common signatures. Unique BLIMP-1 expression signatures in cases wild type for TP53 included MS4A1/CD20, LAX1, IG1, PRF1, DNAJ5, DNAJ9, MAV1, MOV10, LANC12, CD28BP and IRF1. In the TP53-mutated subgroup, STAT3 which is a BCL6 target gene was downregulated in BLIMP-1− cases. Other unique signatures in DLBCL with mutated TP53 included CFLAR, RBP1, CMAH, DNAJC13, DLEU2, VAMP5, and many others.

PRDM1 mutations within exon 1/2, but not mutations in exons 3–7, showed gene expression signatures
We further compared GEP between mutated and wild-type PRDM1 DLBCLs regardless of BLIMP-1 expression. In the overall DLBCL and GCB subset, 103 and 193 DEGs were identified with FDR thresholds of 0.10 and 0.05, respectively. No DEGs were identified in the ABC subset, however, probably because of heterogeneity. Similarly, GEP comparisons between cases with mutated PRDM1 exon 1/2 and those with wild-type PRDM1 only identified DEGs in

| Variables | Total | ABC-DLBCL | GCB-DLBCL | Overall DLBCL |
|-----------|-------|-----------|-----------|---------------|
| Patient no. | 520 | n = 92 | n = 158 | n = 132 | n = 388 |
| ABC subtype | 518 | — | — | — | — |
| Ki-67 ≥ 70% | 516 | 76% (70/92) | 65% (102/158) | 56% (22/39) | 60% (136/225) | 0.72 |
| BCL2 translocation | 417 | 3.8% (3/80) | 4% (5/127) | 2% (8/36) | 35% (60/173) | 0.17 |
| Bcl-2 ≥ 70% | 514 | 55% (50/91) | 59% (93/157) | 28% (11/39) | 47% (96/205) | 0.11 |
| MYC translocation | 332 | 9% (6/70) | 7% (7/101) | 3% (1/37) | 18% (24/130) | 0.049 |
| MYC ≥ 70% | 513 | 31% (28/91) | 42% (66/165) | 18% (7/39) | 29% (65/223) | 0.15 |
| BCL6 translocation | 362 | 44% (30/69) | 39% (39/101) | 40% (12/31) | 23% (36/159) | 0.06 |
| Bcl-6 ≥ 50% | 510 | 52% (47/90) | 49% (77/157) | 66% (26/39) | 76% (17/224) | 0.23 |
| TP53 mutation | 459 | 13% (10/80) | 21% (30/142) | 16% (6/38) | 30% (59/198) | 0.077 |
| p53 ≥ 20% | 450 | 39% (31/80) | 33% (46/139) | 26% (10/38) | 38% (75/193) | 0.20 |
| Wild-type p53 ≥ 20% | 348 | 36% (25/70) | 20% (22/110) | 28% (9/32) | 26% (35/136) | 0.82 |
| Mutated p53 ≥ 20% | 102 | 60% (6/10) | 83% (24/29) | 17% (1/6) | 70% (40/57) | 0.017 |
| p63 ≥ 10% | 499 | 51% (45/89) | 41% (63/153) | 53% (20/38) | 38% (81/215) | 0.10 |
| MDM2 > 10% | 513 | 55% (50/91) | 34% (15/44) | 46% (18/39) | 34% (77/225) | 0.16 |
| p21 > 5% | 450 | 40% (32/80) | 38% (59/139) | 42% (16/38) | 19% (26/133) | 0.0028 |
| IRF4/MUM1 > 30% | 515 | 84% (76/91) | 73% (115/158) | 35% (14/39) | 22% (51/227) | 0.062 |
| GCT1 > 50% | 511 | 19% (17/90) | 18% (13/75) | 38% (15/39) | 54% (72/132) | 0.084 |
| CD10 > 30% | 517 | 10% (9/92) | 7% (7/118) | 64% (25/39) | 70% (159/228) | 0.57 |
| Nuclear p65+ | 467 | 65% (53/84) | 50% (75/149) | 62% (21/34) | 61% (121/198) | 1.0 |
| Nuclear p50 > 20% | 452 | 46% (36/79) | 39% (55/141) | 37% (13/35) | 26% (50/196) | 0.16 |
| pAKT > 30% | 493 | 51% (45/88) | 34% (51/151) | 51% (19/37) | 37% (79/215) | 0.1 |
| CD30+ | 516 | 23% (21/92) | 10% (15/156) | 21% (12/58) | 14% (52/372) | 0.082 |

Abbreviations: ABC, activated B-cell-like; DLBCL, diffuse large B-cell lymphoma; GCB, germinal center B-cell-like. Bold values are the significant P values or those showing trends of significance.
**Figure 4.** Gene expression profiles for the overall and ABC-DLBCL patients and the BLIMP-1 network in DLBCL. (a) Differential expression of genes between patients with and without homozygous PRDM1 deletions, and between patients with and without BLIMP-1 protein expression. Differential expression of genes were only found between wild-type BLIMP-1+ and BLIMP-1−DLBCLs but not between mutated BLIMP-1+ and BLIMP-1−DLBCLs. (b) A brief network of BLIMP-1’s functions and regulations summarizing our results. BLIMP-1 can be activated by p53, IRF4 and NF-κB signaling. BLIMP-1 represses the transcription of MYC, B-cell antigen/surface receptors and germinal center programs, whereas activates LAX1, which inhibits BCR signaling; therefore, leading to attenuated BCR signaling and decreased tumor cell proliferation. BLIMP-1 also transactivates XBP1 and ELL2, which results in activation of plasmacytic differentiation and immunoglobulin production.
| Function category                                      | Homozygous PRDM1 deletion-positive vs -negative DLBCL | Overall BLIMP-1 protein-negative vs -positive DLBCL | Wild-type BLIMP-1 protein-negative vs -positive DLBCL |
|-------------------------------------------------------|-------------------------------------------------------|------------------------------------------------------|-------------------------------------------------------|
|                                                        | Overall DLBCL | ABC-DLBCL | Overall DLBCL | ABC-DLBCL | Overall DLBCL | ABC-DLBCL |
| Cytokine, immune/inflammation related                 | FDR < 0.1    | FDR < 0.3 | FDR < 0.01    | FDR < 0.05 | FDR < 0.01    | FDR < 0.3 |
| Up NLRP7, IL10                                        | NLRP7        |          |              |          |              |          |
| Down —                                                | —            |          |              |          |              |          |
|                                                        |              |          | GZMB, SLAMF7, PRF1, IL2RB, GBP1, GZMA, APOBEC3B, GBP5, GBP2, FAM46C, Stat1, FCER1G, CIQC, IRF1, SRGN, CIorf38, RAB27A, IDO1, C7ST, CCR5, IL21R, HLA-A, HLA-B, IRF2, NLRCS, B2M, ZBP1, MGC29506, ZC3HAV1, TAP1 | —          | —          | —          |
| Differentiation                                        | FDR < 0.1    | FDR < 0.3 | FDR < 0.01    | FDR < 0.05 | FDR < 0.01    | FDR < 0.3 |
| Up DPPA4                                              | —            | SLFN5     |              |          |              |          |
| Down —                                                | —            |          | BCL11A, BATF  |          |              |          |
| B/T antigen/surface receptor and regulation           | FDR < 0.1    | FDR < 0.3 | FDR < 0.01    | FDR < 0.05 | FDR < 0.01    | FDR < 0.3 |
| Up —                                                  | —            |          | MS4A1, CD22, GCNT2, BLK | CD22, CD37 | MS4A1, GCNT2 | CD37 |
| Down —                                                | —            |          | TARP, TRGC2   |          | LAX1         | —          |
| Transcription factor, RNA processing, protein synthesis, folding, transportation and endoplasmic reticulum stress | FDR < 0.1    | FDR < 0.3 | FDR < 0.01    | FDR < 0.05 | FDR < 0.01    | FDR < 0.3 |
| Up STAT3, HLF, TCF19, TSHZ1, HMGa2                    | HLF, ZNF684, ELL3, SERINC4, PLEKHF2, TEAD2, DNAJC10 | —          | ELL3 // SERINC4, DNAJC10, PLEKHF2, TEAD2, DNAJC10 | —          | GOLGA8B    |
| Down —                                                | —            |          | PRDM1, ELL2, XPB1, HSP90B1, FKB11, CRTCL3, WARS, RUNX3, SUB1, 1R1N, SSR4, DNAJC3, CT5B, VAMPS, NEAT1, SAR1B, DNAJ89, LAP3, ARID3A, LITAF, SEC11C, SSR1, SSR3, ARL4C, RALB, RNF149, MANF, HSPA13, C1orf24, SEC24A, PRF1, RAB27A, IDO1, C7ST, CCR5, IL21R, HLA-A, HLA-B, IRF2, NLRCS, B2M, ZBP1, MGC29506, ZC3HAV1, TAP1 | — | — |
| Cell cycle, DNA replication, chromatin structure, DNA damage response, proliferation and signaling | FDR < 0.1    | FDR < 0.3 | FDR < 0.01    | FDR < 0.05 | FDR < 0.01    | FDR < 0.3 |
| Up NLRP2, CDKN2AP, EPHA4, SMARCA1, C1orf2, PRM1       | NLRP2, TRIP13, CDKN2AP, CENPL, EPHA4, RAB23, RG54 | —          | PRDM1, UBE2J1, FKB11, ZBTB38, XPB1, HERPUD1, DNAJC3, SEC24A, HSPA13, ERLEC1, MANF, EIF2AK3, DNAJB9 | — | — |
| Down —                                                | —            |          | DUSP4, MIR21, VDR, CDKN1B, SERP1NA, SPRY4, PIM2, GIMAP6, TMEM184B, PIKP3S, GRIN3A, CALM1 | DUSP4, VDR, TRIB1 | — | VDR |
| Cell death and tumor suppression                       | FDR < 0.1    | FDR < 0.3 | FDR < 0.01    | FDR < 0.05 | FDR < 0.01    | FDR < 0.3 |
| Up TNFAIP8                                            | —            |          | PAWR, TXNDC5, CFLAR, TMEM49, MLKL, MCL1, GADD45A, GHTM, ARMCC3 | — | — | MEG3 |
| Down —                                                | —            |          | TXNDC5, GHITM |          | MLKL, TXNDC5, CFLAR | TXNDC5, TMEM59 |
| Metabolism, autophagy and oxidative stress            | FDR < 0.1    | FDR < 0.3 | FDR < 0.01    | FDR < 0.05 | FDR < 0.01    | FDR < 0.3 |
| Up CYB5R2, SLCA2A13, LPCAT4, ELOV4.7, TGDS, MOCS, LPCAT4 | CYB5R2, SLCA2A13, LPCAT4, ELOV4.7, TGDS, MOCS, LPCAT4 | —          | ATP8B1, SLC39A9 | — | ACSM1, SLC39A9 |
| Down —                                                | —            |          | CHST2, WIP1, TMEM59, SOD2, TXN, GLUL, ACP2, P4HB, SLC39A8, GLA, SAT1, GALM, GSS, APOL6, GLRX, ACSL1 | GSS, TMEM59 | — | — |

Table 4. Differentially expressed genes between homozygous PRDM1 deletion-positive and -negative DLBCL and between BLIMP-1 protein-negative and -positive DLBCL
### DISCUSSION

In this study, we analyzed the clinical and experimental data on 520 *de novo* DLBCL cases to determine the tumor-suppressive function of PRDM1/BLIMP-1. Previous studies demonstrated that loss of PRDM1/BLIMP-1 function is critical for the pathogenesis of ABC-DLBCL.\(^8,11\) Herein, we provide evidence that loss of PRDM1/BLIMP-1 function is a factor for poor prognosis in ABC-DLBCL. We found homozygous PRDM1 deletions and PRDM1 mutations within PRDM1 exons 1 and 2 were poor prognostic factors in patients with ABC-DLBCL. Loss of BLIMP-1 protein expression was common in ABC-DLBCL and associated with a decreased plasma-cell differentiation signature and upregulation of B-cell antigens.

In univariate and multivariate analyses, we found that the prognosis for DLBCL with homozygous PRDM1 deletion was worse than that for DLBCL without this deletion. However, this homozygous deletion only had prognostic significance in the ABC subgroup, as its impact on prognosis for GCB-DLBCLs was minimal. Although the number of patients harboring homozygous PRDM1 deletions was small, our GEP still supported reduced transcriptional repression in patients with these deletions as suggested by upregulation of transcription factors, including ZNF385C, HLF and HMGA2. Notably, we only observed these gene expression signatures in the ABC group.

Similarly, PRDM1 mutations only influenced survival of DLBCL in the ABC subgroup. Our data further support this by demonstrating that PRDM1 mutations within exons 1 and 2 still significantly affect survival of ABC- but not GCB-DLBCLs. Therefore, we suggest that intact BLIMP-1 function is critical for the repression of ABC-DLBCLs. Exons 1 and 2 encode for the first 97 amino acids of BLIMP-1, including the N-terminal acidic domain. An intact N-terminal acidic domain is critical for the normal transcriptional repression function of BLIMP-1.\(^3,4\) Researchers showed that BLIMP-1 lacking this domain do not repress the MYC promoter.\(^3,4\) Interestingly, all but two of the PRDM1 mutations identified in our cohort were missense mutations. Mandelbaum et al.\(^11\) proved that a subset of missense mutations of PRDM1 can directly impair BLIMP-1’s protein stability as well as its transcriptional repression function, including 2 missense mutations at P48 (corresponding to 84P in this article due to different transcripts), the most frequently associated with the ABC phenotype of DLBCL.\(^6,35\)

At the protein level, although BLIMP-1 expression was frequently associated with the ABC phenotype of DLBCL, 63% of the ABC-DLBCL patients lacked BLIMP-1 protein expression. Over half of these BLIMP-1-\(^1\) ABC-DLBCL patients had no apparent genetic changes in PRDM1. \(^9,11\) In *vitro* studies have demonstrated that BLIMP-1 can be inactivated by constitutively active Bcl-6.\(^9,11\) We found a negative correlation between Bcl-6 and BLIMP-1 expression with borderline significance. Also, expression of BCL11A, a cofactor with Bcl-6, was upregulated in our GEP analysis of both ABC and GCB subtypes of BLIMP-1-\(^1\) DLBCLs.\(^3,5\)
Additionally, PRDM1 can be epigenetically regulated by diverse miRNAs including miR-23, miR-9 and let-7a. 23–27 Using miRNA profiling, we found upregulation of miR-30d and miR-30b-5p in BLIMP-1+ cases. Further functional evidence are needed to elucidate the regulation between these miRNAs and BLIMP-1 expression.

ABC-DLBCL patients without expression of BLIMP-1 had decreased expression of the tumor suppressor protein p53 and its downstream targets MDM2 and p21, as well as increased expression of Myc, which promotes cell proliferation. 30,31 Therefore, in BLIMP-1+ DLBCL cases, decreased p53 signaling and Myc overexpression may synergistically promote tumor progression. In addition, loss of BLIMP-1 expression was associated with dysregulation of NF-kB pathway molecules. Induction of PRDM1 mRNA expression in B-cell lymphoma cell lines can be blocked by NF-kB inhibitors. 30,31 Recently, Heise et al. 30,31 proved that NF-kB subunit p65 (RELA) is required for the induction of BLIMP-1 expression, which is consistent with the positive association of p65 expression and BLIMP-1+ in our ABC DLBCL cohort.

Surprisingly, BLIMP-1 protein expression was not a survival predictor in the present study. Gyory et al. 30,31 found that the alternative splicing protein BLIMP-1β, which lacks the first three exons in the normal BLIMP-1 protein, does not have transcriptional repression activity. Consistently, detection of BLIMP-1β mRNA expression in tumor cells has been associated with poor prognosis for ABC-DLBCL. 30 Using our immunohistochemical assay, we could not discriminate between the normal BLIMP-1 protein and BLIMP-1β. The presence of BLIMP-1β in our cohort may reduce the prognostic impact of normal BLIMP-1 by diminishing its ability to repress the activity of downstream targets such as Myc and Bcl-6.

However, lack of BLIMP-1 expression seemed to be an unfavorable prognostic factor for DLBCL in patients with aberrant p53 statuses. Kusam et al. 30,31 reported that Bcl-6 can immortalize primary B cells and greatly increase B-cell function only in the absence of normal p53 function. BLIMP-1 is a known transcriptional repressor of BCL6. 30,31 We found that BLIMP-1 positivity was closely associated with the absence of Bcl-6 protein expression in our group of DLBCL patients with mutated TP53. Therefore, BLIMP-1 may inhibit the proliferation of tumor cells harboring mutated TP53 by repressing Bcl-6 activity. Consistently, GEP analysis in DLBCL patients with mutated TP53 found that expression of IRF4 (an activator of BLIMP-1 and inhibitor of BCL6) and STAT3 (a BCL6 target gene for repression), 30,31 were downregulated in the BLIMP-1+ subgroup.

The BLIMP-1 negativity signature in the overall cohort of DLBCL patients was similar to that in the ABC subgroup: both were characterized by downregulation of plasmacytic differentiation and upregulation of B-cell antigens. BLIMP-1’s role in promoting plasmacytic differentiation 27 of DLBCL was indicated by downregulation of XBP1 along with several of its downstream targets involved in protein synthesis in endoplasmic reticulum and transportation in BLIMP-1+ cases. 30,31 XBP1 is a transcriptional activator that acts downstream of BLIMP-1. BLIMP-1 and XBP1 are jointly required for the establishment of terminally differentiated plasma cells. 30,31 Moreover, we found that expression of genes involved in immunoglobulin production (ELL2, MZB1 and ARID3A) and cell differentiation (BATE7) was downregulated in the BLIMP-1+ group. On the other hand, loss of BLIMP-1 expression in DLBCL correlated with upregulation of CD22 and BLK and downregulation of LAX1 that have important roles in regulating BCR signaling. Importantly, expression of MS4A1 (encoding CD20) was also upregulated in BLIMP-1+ cases, possibly indicating an increased therapeutic effect of rituximab in patients with BLIMP-1+ DLBCL as suggested previously. 32,33 In line with the role of BLIMP-1 in plasmacytic differentiation suggested by GEP analysis, the cell-of-origin analysis based on B-cell-associated gene signatures 22 also showed positive correlation between BLIMP-1 expression and the plasmablastic subtype of DLBCL (Table 1).

The antiproliferative effect of BLIMP-1 on DLBCL was also suggested by the GEP results as cell-cycle suppressors (e.g., CDKN1B, GADD45A, GHTIM, DUSP4) were upregulated in BLIMP-1+ DLBCls. Also, in vitro experiments have demonstrated that B-cell proliferation in DLBCL cell lines was promoted upon acute ablation of BLIMP-1 and that BLIMP-1 inactivation led to B-cell lymphomagenesis and shortening of life spans in mouse models of DLBCL. 34,35 Reintroduction of BLIMP-1 into a DLBCL cell line likely represses the cell’s proliferation by turning off MYC and other genes involved in cell-cycle progression and DNA repair. 36 A brief network of BLIMP-1’s functions and regulation generated from our results is presented in Figure 4b.

Collectively, the results of the present study demonstrated that loss of PRDM1/BLIMP-1 function contributed to the overall poor prognosis for ABC-DLBCL in three ways. First, genetic PRDM1 aberrations (including homozygous deletions and mutations) affected the prognosis for ABC- but not GCB-DLBCLs. Second, a lack of BLIMP-1 expression correlated with an impaired p53 signaling pathway and Myc overexpression in ABC-DLBCLs. Third, the GEP signatures of BLIMP-1 expression for the overall DLBCL patient cohort resembled those for the ABC subgroup, both indicating downregulation of plasmacytic differentiation of DLBCls and upregulation of B antigens and BCR signaling in BLIMP-1+ tumors. These data may suggest that inactivated BLIMP-1 facilitates DLBCL progression through Myc and BCR signaling, which are essential for survival of ABC-DLBCL 18,36 Therapeutic approaches that restore the normal function of BLIMP-1 may help drive terminal differentiation of tumor cells and overcome the chemoresistance of ABC-DLBCL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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