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Prognostic value of lymphocyte-to-monocyte ratio and histone methyltransferase G9a histone methyltransferase in patients with double expression lymphoma

A retrospective observational study

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Abstract

In patients with diffuse large B-cell lymphoma, MYC combined with Bcl2 and/or Bcl6-based protein expression is called double expression lymphoma (DEL). R-DA-EPOCH program chemotherapy is typically recommended because these patients often have a poor prognosis. Although numerous factors affect survival of patients with DEL, the roles of the tumor biomarker histone methyltransferase G9a (G9a) and the lymphocyte-to-monocyte ratio (LMR) are unknown.

We performed a retrospective analysis of data from 51 patients. These patients were newly diagnosed with DEL and treated with R-DA-EPOCH at Taizhou People' s Hospital and Northern Jiangsu People's Hospital between June 2014 and December 2019. Receiver operator characteristic curve results were used to calculate the LMR cutoff value. We used an immunohistochemical analysis to examine G9a expression in DEL tissues. The Kaplan–Meier method was used to determine progression-free survival (PFS) and overall survival (OS) characteristics. Cox proportional-hazards models were constructed for univariate and multivariate analyses to examine the prognostic values of LMRs and G9a in patients with DEL.

The cutoff value for LMR was 2.18. The 5-year PFS rate was 35.3%, and the 5-year OS rate was 39.2%. Patients with DEL with lower LMRs and who were G9a-positive predicted inferior PFS and OS. Univariate analysis revealed that patients with elevated LDH levels, high National Comprehensive Cancer Network International Prognostic Index (NCCN-IPI) scores, LMRs $\leq$2.18, and G9a-positive results had relatively poorer PFS and OS. The multivariate analysis revealed that LMRs $\leq$2.18 and a G9a-positive result were independent prognostic factors for PFS and OS in patients with DEL treated with R-DA-EPOCH.

The study results suggested that peripheral blood LMRs were an important marker for evaluation of prognosis in patients with DEL. High expression of G9a was associated with worse outcomes, indicating that G9a may serve as a prognostic biomarker for patients with DEL who undergo R-DA-EPOCH program chemotherapy.

Abbreviations: ABC = activated B-cell-like, DEL = double expression lymphoma, DLBCL = diffuse large B-cell lymphoma, G9a = G9a histone methyltransferase, GCB = germinal center B-cell-like, LMR = lymphocyte-to-monocyte ratio, OS = overall survival, PFS = progression-free survival.

Keywords: diffuse large B-Cell lymphoma, double expression lymphoma, G9a histone methyltransferase, lymphocyte-to-monocyte ratio

1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma. It is highly heterogeneous in clinical manifestation and prognosis. DLBCL accounts for about 30% to 40% of non-Hodgkin lymphomas in the West and is more prevalent in developing countries.[1–3] Long-term survival times of patients with DLBCL have improved significantly with use of the targeted drug rituximab[4–6]; 5-year overall survival has

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

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reached 60% to 70%.[7] However, nearly 30% of patients relapse and experience refractory double expression lymphoma (DEL), even if they accept the second-line regimen. As understanding of the molecular-level pathogenesis improves, more and more molecular subgroups have been identified. In patients with DLBCL, MYC combined with Bcl2 and/or Bcl6-based protein expression is called DEL. This disease is highly invasive, with frequent extranodal invasion and a poor prognosis. Enhanced treatment programs (e.g., R-DA-EPOCH and R-CVAD A/B) are used because the conventional R-CHOP treatment program is ineffective in these patients.[8] Accepted criteria for clinical prognosis remain to be developed.

Study results indicate that the tumor microenvironment is critically important to the initiation and progression of carcinogenesis. This microenvironment can suppress tumor outgrowth by destroying cancer cells or inhibiting their outgrowth. It also promotes tumor progression via tumor cell growth, infiltration, metastasis, and angiogenesis.[9,10] Monocytes and lymphocytes are important components of the immune response and have important roles in formation of the tumor microenvironment. High absolute monocyte counts and low lymphocyte-to-monocyte ratios (LMRs) are associated with decreased progression-free survival (PFS) and overall survival (OS) times in patients with newly diagnosed DLBCL and Hodgkin lymphoma.[11–13]

Epigenetic regulation (e.g., DNA methylation and histone modification) is an important mode of action during tumor initiation and progression.[14–16] G9a (euchromatin histone lys N-methyltransferase 2) is a member of the Suv39h protein family with the su(var) 3–9, enhancer-of-zeste and trithorax; shGFP, hairpin targeting green fluorescent protein (SET) domain.[17] G9a is closely related to biologic processes such as tumorigenesis, embryonic development, cognitive and adaptive behavior, and adipogenesis.[18–21] G9a protein is highly expressed in various tumors.[22] In this study, we analyzed the prognostic value of G9a and LMR on PFS and OS characteristics in patients with DEL.

2. Methods

2.1. Patient population

Fifty-one patients with newly diagnosed DLBCL admitted to the People's Hospital of Taizhou and Northern Jiangsu People's Hospital between June 2014 and December 2019 were enrolled in the study. They were diagnosed with DLBCL based pathological biopsy and immunohistochemical staining results. Immunohistochemical expression of MYC >40% and Bcl2 or Bcl6 >50% was defined as DEL. The results of one or more tests (i.e., bone marrow pathology, B-ultrasound of the systemic and superficial lymph nodes, abdomen, and pelvic cavity, CT, Magnetic Resonance Imaging, and Positron Emission Tomography-Computed Tomography) were evaluated to assess the extent of lymphoma systemic involvement. All patients were treated with the R-DA-EPOCH regimen, and complete clinical and follow-up data were collected. Absolute counts of peripheral blood lymphocytes and monocytes were collected from each patient at initial diagnosis using standard automated complete blood counts methods. Tumor staging was performed according to the Ann Arbor staging system. The National Comprehensive Cancer Network International Prognostic Index (NCCN-IPI), which includes age, stage, and LDH (lactate dehydrogenase) level, and the Eastern Cooperative Oncology Group performance status and the site of extranodal invasion were also recorded.

The study was performed in accordance with Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice Guidelines. The Ethical Review Board of the People's Hospital of Taizhou and the Ethical Review Board of Northern Jiangsu People's Hospital approved the study protocol. All patients gave informed consent.

2.2. Chemotherapeutic regimen

All 51 patients were treated with the R-DA-EPOCH regimen: rituximab 375 mg/m^2/d on day 0, etoposide 50 mg/m^2/d, vincristine 0.4 mg/m^2/d, doxorubicin 10 mg/m^2/d on days 1 to 4, cyclophosphamide 750 mg/m^2 on day 5, prednisone 6 mg/m^2 on days 1 to 5, and granulocyte colony-stimulating factor 5 μg/kg/d administered subcutaneously from day 6 until the absolute neutrophil count reached 5×10^9/L.[23] The regimen was repeated every 3 weeks; the median treatment cycle was 4 times.

2.3. Measurement of G9a expression using immunohistochemical assay

Immunohistochemistry was used to evaluate G9a expression in lymphoma tissue samples acquired from the 51 patients. The samples were fixed in 4% paraformaldehyde and embedded in paraffin embedded by standard methods. Antigen retrieval was performed with citrate buffer (10 mM citric acid-0.05% Tween 20, pH 6.0). Tissue sections were stained using standard immunohistochemistry techniques, using antibodies as indicated; a 1 g/L hematoxylin solution was used for counterstaining. The primary antibodies used were rabbit monoclonal G9a antibody (1:100 dilution, ab133482, Abcam, Cambridge, MA), II goat anti-rabbit biotinylated IgG in phosphate-buffered saline containing 1% bovine serum albumin for 30 minutes at ambient temperature, and then incubated with avidin-biotin complex reagent for 30 minutes. Immunostaining results were visualized using 3,30-diaminobenzidine. Slides were dehydrated via graded alcohol to xylene immersion and mounted with coverslips. Hematoxylin was used for nuclear counterstaining. G9a expression was assessed by 2 pathologists without any knowledge of the patients' information. The positive G9a expression cells exhibited to have brownish-yellow granules in their nucleus. G9a expression was graded based on the following standards: <10% of lymphoma cell population unstained or stained was recorded as a negative result; ≥10% of lymphoma cell population stained was recorded as a positive result.[22]

2.4. Statistical analysis

Receiver operator characteristic (ROC) curve analysis was performed to calculate the cutoff value for LMR. The Kaplan–Meier method was used to determine PFS and OS characteristics. PFS time was defined as the duration from diagnosis to disease progression, death, or the last follow-up. OS time was defined as the duration from diagnosis to death or the last follow-up. Survival curves were generated using the Kaplan–Meier method. Cox proportional-hazards models were constructed for univariate and multivariate analyses to evaluate prognostic effects. Log-rank tests were used to estimate the significance of statistical differences. All two-sided P-values $<.05$ were considered to be statistically significant. All statistical analyses were performed using SPSS 23.0 (SPSS Inc, Chicago, IL) or GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA) software.

3. Results

3.1. Patient characteristics

We retrospectively analyzed data from 51 DEL patients in this study. Results for the clinical features of all patients are presented in Table 1. The median age at diagnosis was 58 years (range, 44–73 years). Of these, 52.9% and 47.1% were ≤60 years or >60 years of age, respectively; 33.3% and 66.7% were classified with stage I–II or stage III–IV disease, respectively. In this patient population, 41.2% had germinal center B-cell-like (GCB) cell of origin tumors and 58.8% had activated B-cell-like (ABC) cell of origin tumors. Based on NCCN-IPI score, 42 patients (82.3%) were in the low-risk and low-intermediate-risk group (0–3 points) and 9 patients (17.7%) were in the high-intermediate-risk and high-risk group (≥4 points). Immunohistochemical analysis revealed that G9a was positive in 64.7% and negative in 35.3% of the lymphoma samples (Fig. 1).

Table 1

Demographic and clinical characteristics of study subjects.

Variable	Characteristics	Total number (%)
Age, y		
≤60	27	52.9
>60	24	47.1
Sex		
Male	25	49
Female	26	51
Stage		
I–II	17	33.3
III–IV	34	66.7
NCCN-IPI score		
≤3	42	82.3
>3	9	17.7
B symptoms		
Present	11	21.6
Absent	40	78.4
Extranodal sites		
0–1	41	80.3
≥2	10	19.7
LDH		
≤250 U/L	15	29.4
>250 U/L	36	70.6
COO		
GCB	21	41.2
ABC	30	58.8
G9a		
Positive	33	64.7
Negative	18	35.3

ABC = activated B-cell-like; COO = cell of origin; G9a = G9aHistone methyltransferase; GCB = germinal center B-cell-like.

3.2. Cutoff value of LMR

At diagnosis, the median absolute lymphocyte count was 1.54×10^9/L (range, 0.52–3.63); the median absolute monocyte count was 0.63×10^9/L (range, 0.23–1.68). The most discriminative cutoff value of LMR obtained from the ROC curve analysis was 2.18, with an area under the curve value: 0.937, 95% confidence interval (CI): 0.875–0.999 ($P < .001$), where the ratio ≤2.18 was 26 (51%) cases, and the ratio >2.18 was 25 cases (49%) (Fig. 2).

3.3. Survival analysis

The median follow-up time following diagnosis was 37 months for the entire cohort (range, 6–60 months). The 5-year PFS rate was 35.3%, and the 5-year OS rate was 39.2%. The 5-year PFS of patients in the peripheral blood LMR ≤2.18 group was significantly lower than that in the LMR >2.18 group ($P = .01$) (Fig. 3A). Kaplan–Meier analysis revealed that a lower LMR at diagnosis was associated with an inferior 5-year OS ($P = .003$; Fig. 3B). At the same time, the 5-year PFS and OS of patients with G9a-positive tumors were significantly worse than for patients with G9a-negative tumors (PFS: $P < .001$, Fig. 3C; OS: $P < .001$, Fig. 3D). Patients in the high-risk group identified using NCCN-IPI had dismal outcomes, with 5-year PFS and 5-year OS rates of 11%. In contrast, low-risk patients experienced a 5-year PFS of 40.5% and a 5-year OS of 45.2% (PFS: $P < .001$, Fig. 3E; OS: $P < .001$, Fig. 3F).

3.4. Univariate analysis and multivariate analyses

Univariate analysis revealed that patients with elevated LDH levels, high NCCN-IPI scores, LMRs ≤2.18, and G9a-positive tumors experienced relatively poorer PFS and OS times (Table 2). Factors with a $P < .05$ value in the univariate analyses were

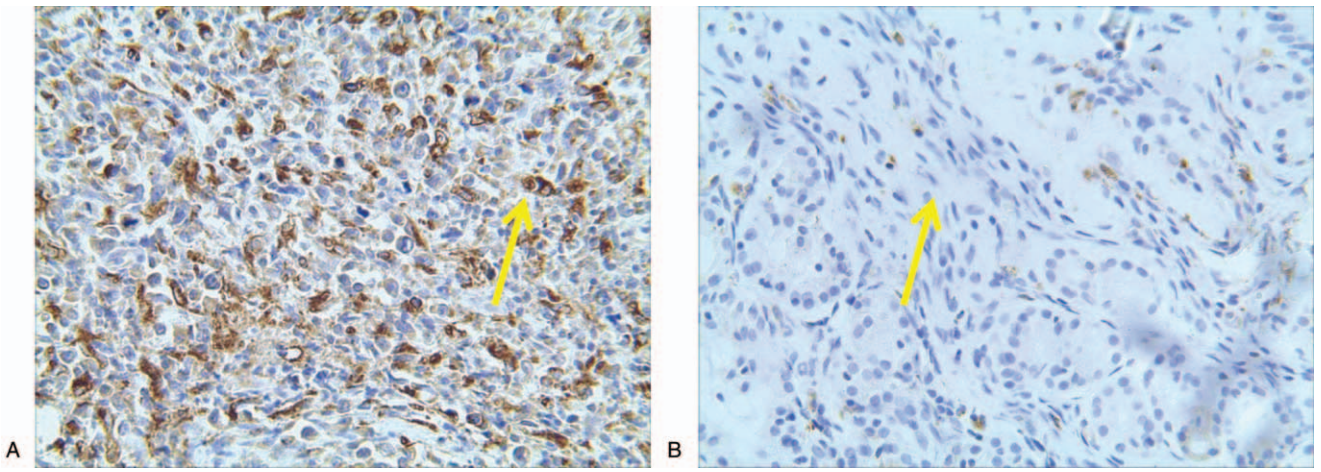

Figure 1. Immunohistochemical analysis of G9a expression. A, G9a-positive. B, G9a-negative. ×100 magnification.

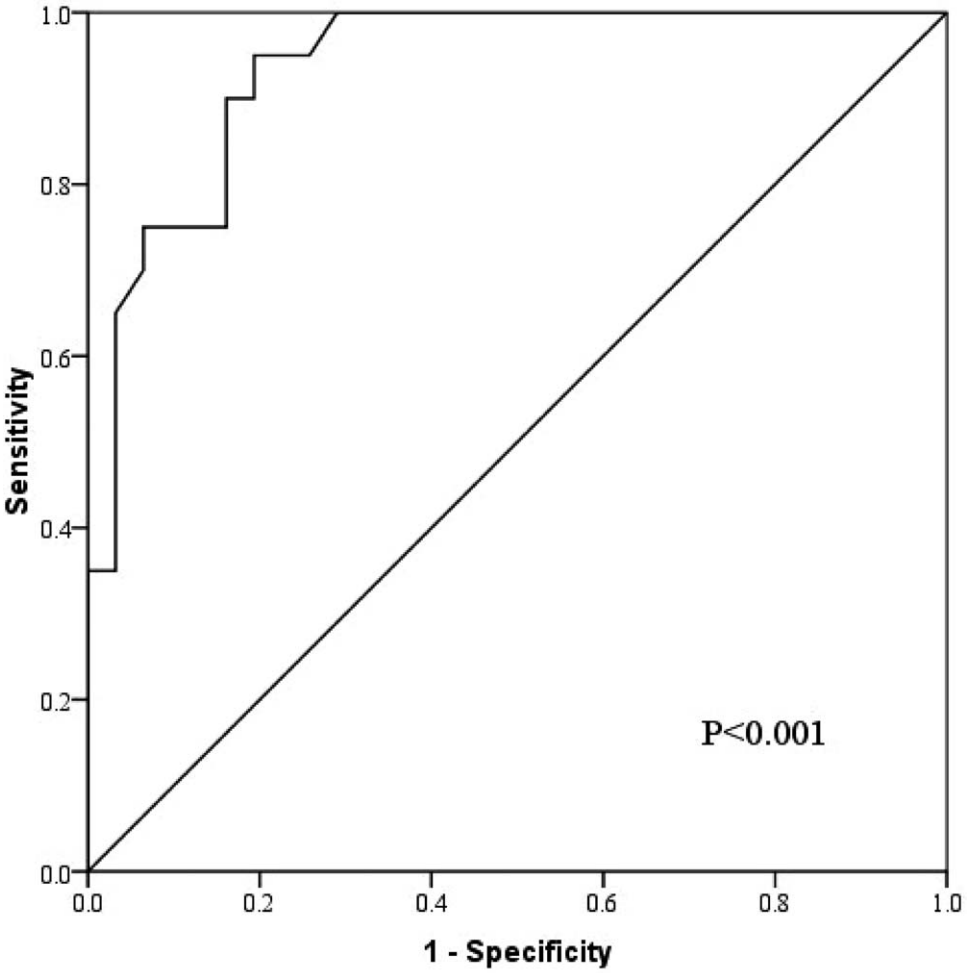

Figure 2. Receiver operating characteristic (ROC) curve analysis of lymphocyte-to-monocyte ratios.

included in the multivariate analysis. Of these, an LMR ≤2.18 and a G9a-positive tumor were identified as independent prognostic factors for PFS (LMR: $P=.01$; G9a: $P=.02$) and OS (LMR: $P=.01$; G9a: $P=.04$) in patients with DEL treated with R-DA-EPOCH. Among the other variables studied, LDH level was shown to be an independent predictor for PFS, while NCCN-IPI independently predicted inferior OS (Table 3).

4. Discussion

DLBCL in which MYC expression exceeds 40% and Bcl2 or Bcl6 expression exceeds 50% based on immunohistochemistry results is defined as DEL. This disease is highly heterogeneous in both histopathological characteristics and clinical manifestations. Previous clinical studies have shown that patients with DEL-associated highly aggressive B-cell lymphoma tend to be older, staged later, and have higher proportions of extranodal invasion and non-GCB pathology types. These factors combine to worsen the overall clinical prognosis.[24] R-DA-EPOCH which can make tumor cells display less resistance to a prolonged drug exposure is suitable for older and highly aggressive DLBCL patients, and durable remission and superior PFS have been observed in selected group.[25–27] NCCN-IPI is currently recognized as a prognostic indicator for DLBCL, which we also found in this study. However, it has limitations when used to predict the prognosis of patients with DEL. Therefore, in this study we examined potential prognostic factors other than NCCN-IPI.

Univariate analysis found that elevated LDH levels, high NCCN-IPI scores, LMRs ≤2.18, and G9a-positive tumors indicated a worse prognosis. We calculated the cutoff value of LMR from the ROC curve as 2.18. Meanwhile, we found that the 5-year PFS of patients with peripheral blood LMRs ≤2.18 was significantly lower than that of patients with LMRs >2.18. Studies found that low LMRs have important predictive value for a variety of solid tumors and for a variety of lymphomas, such as DLBCL, follicular lymphoma, and angioimmunoblastic T-cell lymphoma.[28–34] Belotti et al[35] found that using a combined chemotherapy regimen with rituximab, the 2-year PFS of patients with DLBCL and LMRs <2.4 is significantly lower than that in patients with LMRs ≥2.4. Patients with follicular lymphoma and LMRs <2 also have a worse prognosis. Matsuki et al[32] obtained an LMR cutoff value of 4.7 for follicular lymphoma, and considered it an independent risk factor for prognosis. Koh et al[36] found that in patients with DLBCL treated with R-CHOP, the LMR value significantly affects OS and PFS. The mechanism via which LMR affects the prognosis of patients with malignant tumors is unclear. The decrease in LMR indicates that lymphocytes are relatively reduced and mononuclear cells are

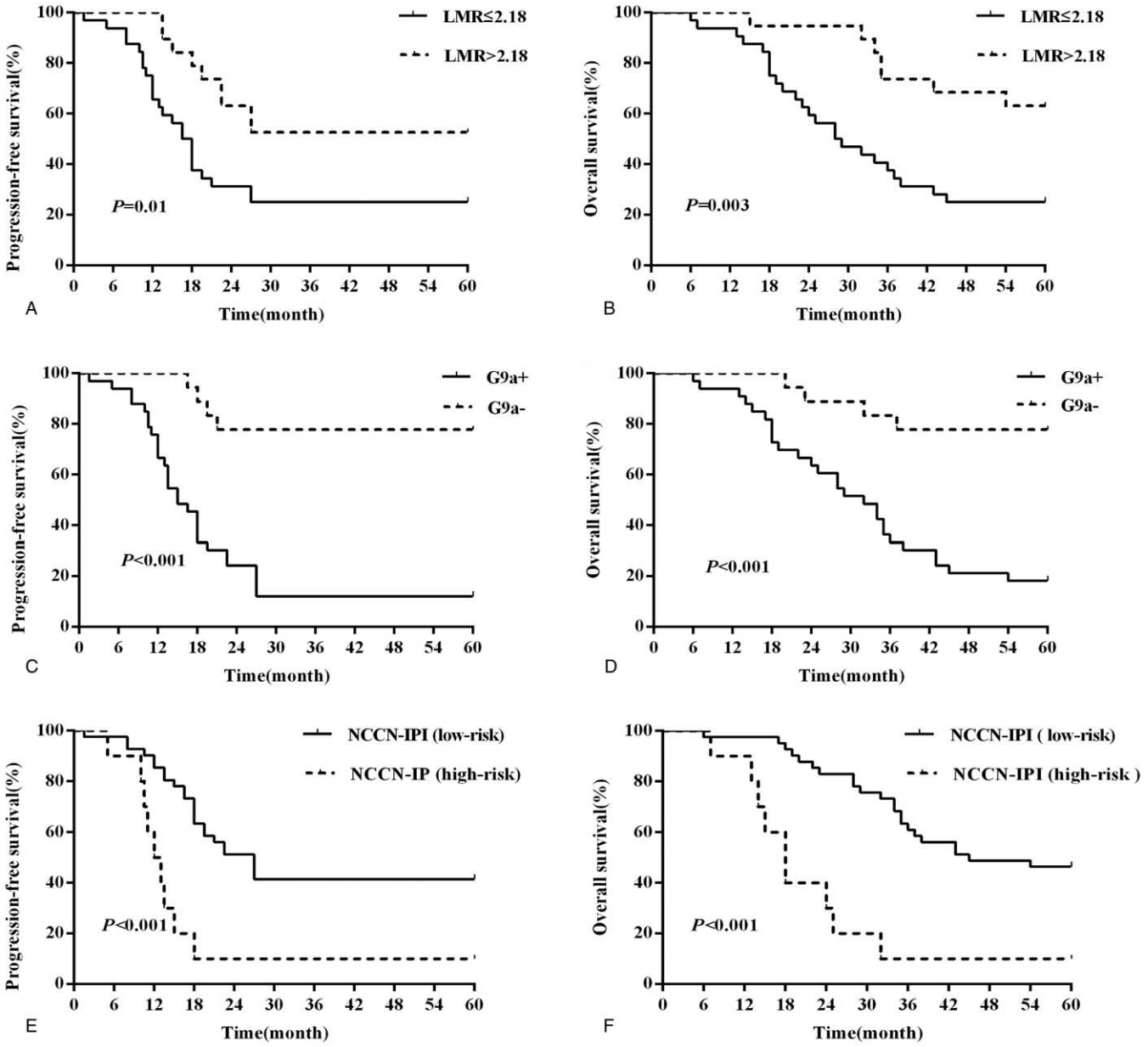

Figure 3. Kaplan–Meier survival analysis of LMR and G9a at diagnosis in patients with DEL treated with R-DA-EPOCH. A, Progression-free survival according to baseline LMR results. B, Overall survival according to baseline LMR results. C, Progression-free survival according to G9a results. D, Overall survival according to G9a results. E, Progression-free survival according to NCCN-IPI results. F, Overall survival according to NCCN-IPI results. DEL = double expression lymphoma; LMR = lymphocyte-to-monocyte ratio; NCCN-IPI = National Comprehensive Cancer Network International Prognostic Index.

relatively increased. LMR can simply and effectively reflect the biological status of immune homeostasis and the tumor microenvironment. Lymphocytes are an important part of human humoral immunity and cellular immunity. They inhibit tumor cell growth, proliferation, and metastasis, and promote tumor cell apoptosis. Monocytes in the circulation can be recruited to tumor tissues by various tumor-secreted chemokines.[37]

To examine the predictive value of the LMR and G9a, they were included in a multivariate analysis. The results indicated that both LMR and G9a were independent factors that affected patients with newly diagnosed DEL; patients with high LMRs and G9a-negative tumors had a better prognosis. We focused on G9a, which catalyzes dimethylation of lysine residue 9 of histone H3.[38] It mediates cell differentiation, proliferation, and gene expression, and patients' responses to drugs. Because it has a vital role in cell proliferation, we reasoned that G9a is involved in tumorigenesis. Overexpression of G9a has been found in many cancers.[39] Bai et al's results suggested that G9a can serve as a prognostic biomarker for hepatocellular carcinoma after surgical resection.[22] Hu et al[40] found that G9a expression is significantly related to the survival time of patients and is an independent risk factor for patients with bladder cancer. In our study, patients with high G9a expression had a poorer prognosis, compared with patients with low expression. This result suggested that G9a can be used as a biomarker for the prognosis

Table 2

Univariate analysis of prognostic factors for survival in DEL patients.

	PFS		OS	
Prognostic factors	**HR (95%CI)**	***P* value**	**HR (95%CI)**	***P* value**
Gender (male vs female)	0.69 (0.348–1.369)	.29	0.827 (0.431–1.768)	.70
Age (>60 vs ≤60)	0.993 (0.500–1.972)	.98	1.4 (0.692–2.834)	.35
Serum LDH level (elevated vs normal)	3.585 (1.377–9.335)	.01	2.978 (1.139–7.783)	.03
ECOG PS (≥2 vs <2)	1.637 (0.824–3.251)	.16	1.325 (0.665–2.682)	.43
Ann Arbor stage (III–IV vs I–II)	1.846 (0.855–3.985)	.12	1.629 (0.749–3.542)	.22
Extranodal sites (≥2 vs <2)	6.518 (2.849–14.909)	.14	1.654 (0.761–3.597)	.20
NCCN-IPI score (>3 vs ≤3)	5.888 (2.622–13.223)	<.001	8.592 (3.723–19.872)	<.001
B symptoms (present vs absent)	0.811 (0.352–1.870)	.62	0.798 (0.327–1.948)	.62
COO (GCB vs ABC)	1.168 (0.588–2.32)	.66	1.4 (0.69–2.842)	.35
LMR (≤2.18 vs >2.18)	1.92 (0.902–4.809)	.04	10.358 (4.056–20.353)	.04
G9a (positive vs negative)	7.466 (2.605–21.367)	<.001	6.185 (2.147–17.813)	.01

HRs was obtained from Cox proportional hazard model. ABC = activated B-cell-like; CI = confidence interval; COO = cell of origin; ECOG PS = Eastern Cooperative Oncology Group performance status; G9a = G9aHistone methyltransferase; GCB = germinal center B-cell-like; HR = hazard ratio; LMR = lymphocyte-to-monocyte ratio; OS = overall survival; PFS = progression-free survival.

Table 3

Multivariate analysis of prognostic factors for survival in DEL patients.

	PFS		OS	
Prognostic factors	**HR (95% CI)**	***P* value**	**HR (95% CI)**	***P* value**
Serum LDH level (elevated vs normal)	3.046 (1.12–28.288)	.03	2.248 (0.81–6.24)	.12
NCCN-IPI score (>3 vs ≤3)	2.291 (0.974–5.391)	.06	4.589 (1.718–12.253)	.01
LMR (≤2.18 vs >2.18)	4.008 (2.007–11.519)	.01	6.092 (2.178–17.04)	.01
G9a (positive vs negative)	5.589 (1.897–16.462)	.02	5.066 (1.631–15.739)	.04

HRs was obtained from Cox proportional hazard model. CI = confidence interval; G9a = G9aHistone methyltransferase; HR = hazard ratio; LMR = lymphocyte-to-monocyte ratio; OS = overall survival; PFS = progression-free survival.

of patients with DEL who undergo R-DA-EPOCH treatment. The uniqueness of this study is that G9a can be used as an independent risk factor for a poor prognosis in patients with DEL. This may be due to the fact that G9a can catalyze the monomethylation and dimethylation of H3K9 (H3K9me1 and H3K9me2) in autochromatin. By inhibiting specific types of methylation of histones in the promoter region of the gene, or with DNA methylation, it can inhibit specific functions of tumor suppressor gene transcription.[41]

There is no recognized standard for treatment of patients with DEL. One study found that the 5-year PFS of patients with DEL who undergo R-CHOP treatment is 20% to 30%.[42] In contrast, our study found that the 5-year PFS after R-DA-EPOCH treatment was >30%. This difference may indicate that use of R-DA-EPOCH or a more intensified regimen can improve the prognosis of these patients. We found that patients with DEL and low LMRs and G9a-positive tumors had a worse prognosis. An alternative regimen such as lenalidomide combined with R-CHOP can be used for these patients.[43,44] New targeted drugs, such as histone deacetylase inhibitors[45,46] and the Bcl-2 inhibitor venetoclax (ABT-199) can also be used. Single-agent ABT-199 has modest anti-tumor activity against most DLBCL lines and results in compensatory up-regulation of MCL1 expression. ABT-199 synergizes strongly, however, when combined with dinaciclib and with other drugs affecting MCL1, including standard DLBCL chemotherapy drugs.[47] An in vitro study found further evidence of synergistic growth suppression by ibrutinib and ABT-199 in multiple ABC-DLBCL, GCB-DLBCL, and follicular lymphoma cell lines.[48]

In conclusion, this study found that the prognosis of patients with DEL after initial treatment could be estimated simply and rapidly based on LMR results. This approach will be helpful for assessment of the patient's systemic immunity and tumor microenvironment. The study results suggested that G9a was associated with cancer progression and prognosis and can serve as a novel therapeutic target. However, a large sample size, multicenter prospective study is needed for further support of these findings.

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Author contributions

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Investigation: Xiaolin Sun, Yaqian Peng.
Methodology: Pei Xu.
Supervision: Jianfeng Zhu.
Writing – original draft: Pei Xu, Xiaolin Sun, Xuyan Song.
Writing – review & editing: Pei Xu, Jianfeng Zhu.

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