ORIGINAL ARTICLE

High Ki67 proliferation index but not cell-of-origin subtypes is associated with shorter overall survival in diffuse large B-cell lymphoma

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ABSTRACT

Background: CD10, BCL6, and MUM1 are commonly used immunohistochemical stains for classifying diffuse large B-cell lymphoma (DLBCL), which is useful in predicting outcome. Conflicting reports of the prognostic value of other markers such as BCL2, CD23, and Ki67 proliferation index have been reported. Our objective was to correlate these immunostains and Hans classification with response to therapy and overall survival. **Materials and Methods:** A retrospective study of patients diagnosed with DLBCL from 2008–2014 at a tertiary-care cancer hospital. The slides with the IHC stains were reviewed by two independent pathologists. The clinical outcomes—assessed independently—were response to therapy and overall survival. The treatment response evaluation was based on the new Lugano classification. Statistical analyses were conducted using the Fisher's exact test and Kaplan–Meier survival curves. Significance was set at $P < 0.05$. **Results:** Forty-one patients were included in the study with a known Hans classification, available clinical data, and at least 5-year follow-up. CD10 immunostain was reported in all patients, whereas CD23 was the least reported in only four patients. No significant association was observed between CD10, BCL6, MUM1, BCL2, and both Response to therapy and overall survival. Owing to few cases reported CD23 immunostain, further analysis of association is not reported. High Ki67 proliferative index of >80% was statistically significantly associated with shorter overall survival and not statistically significant associated with no response to therapy. Hans classification subtypes were not predictive in regard to therapy response. **Conclusion:** High Ki67 expression (>80%) was associated with shorter overall survival in DLBCL. Hans classification subtypes were not predictive.

Key words: Activated B-cell (ABC), BCL2, BCL6, B-lymphocytes, CD10, CD23, cell-of-origin classification, diffuse large B-cell lymphoma (DLBCL), germinal center B-cell (GCB), immunohistochemistry, Ki67, MUM1, overall survival, therapy response

INTRODUCTION

Diffuse large B-cell lymphoma, non-otherwise specified (DLBCL, NOS) is the most common type of adult non-Hodgkin lymphoma worldwide.[1] The standard first-line treatment is multiagent chemoimmunotherapy such as

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R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone). However, it is only curative in approximately 60% of patients.[2] In patients who develop relapsed/refractory disease, stem cell transplantation (SCT) is a considered treatment but many patients are not eligible.[3] A better understanding of the pathophysiology and classification of the DLBCL may help in therapy selection and impact treatment outcomes.

Decades ago, the Kiel classification of DLBCL was based on morphology such as centroblastic, immunoblastic, and anaplastic variants, with centroblastic being the most common.[4] This subtyping was plagued by poor intraobserver and interobserver reproducibility. In the early 2000s, immunophenotypic subtyping was reported then proposed by several groups, most commonly Hans and his colleagues in 2004 who classified DLBCL into two general categories: germinal center B cell (GCB) and activated B-cell (ABC) subtype. The current most widely used working algorithm, also known as the Hans criteria, incorporates three immunostains: cluster of differentiation 10 (CD10), B-cell lymphoma 6 (BCL6), and multiple myeloma oncogen1 (MUM1) with a cutoff of 30% to identify positivity in each.[5] The World Health Organization (WHO) classification of hematopoietic and lymphoid tumors in 2008[6] and later the 2016 WHO revision[1] incorporated the Hans criteria as follows: GCB subtype (CD10+, BCL6 +/−, MUM1 +/− and CD10−, BCL6 +, MUM1−) and ABC subtypes for all other interpretations[7] [Figure 1]. GCB subtype is generally of favorable prognosis compared to ABC profile.[8] However, some studies have shown conflicting results using the Hans classification and showed no significant difference in overall survival between the GCB and ABC.[7] In spite of these efforts, it is not uncommon to encounter lymphomas that do not fit into any of the prescribed categories or do not manifest clinically as would be predicted according to its respective subtype. This discrepancy highlights the heterogeneous nature of large B-cell lymphomas and the need to improve our current classification and prognostication. Additional prognostic markers have been studied in the DLBCL such as BCL2,[9] CD23,[10] and Ki67[11] with conflicting results reported in the literature.

In this study, we examined the predictive value of the Hans classification and markers in a DLBCL patient population, investigated the potential predictive value of two additional markers CD23 and BCL2, and attempted to better integrate Ki67 into the working algorithm.

MATERIALS AND METHODS

For this retrospective study (IRB 2016–207, 12/2/2016), a natural language search was performed in our database information system (Copath) to retrospectively review all adult patients diagnosed with DLBCL, NOS, during the period 2008 to 2014 at Karmanos Cancer Institute, Wayne State University, Detroit, Michigan, an academic tertiary-care cancer hospital. This particular time frame was used to limit the diagnosis and the performed immunohistochemistry (IHC) panels after the publication of the 2008 WHO classification of hematopoietic and lymphoid tumors and to allow the aimed follow-up in the study. The inclusion criteria included: DLBCL diagnosis with a known cell of origin (COO) subtype using Hans criteria, available clinical data and at least 5-year follow-up (unless limited by patient death). Cases of unclassifiable B-cell lymphoma with features intermediate between DLBCL and Burkitt lymphoma or DLBCL and classic Hodgkin lymphoma were excluded.

The tissue samples underwent a lymphoma protocol that is standardized and used in our institute. The immunohistochemical stains were performed by routine methods in the clinical laboratory. The paraffin blocks were cut at 3–4 microns, dried overnight at 60°C, and deparaffinized in xylene. Subsequently, sections were rehydrated through graded alcohol in water. Heat-induced epitope retrieval was done automatically on Ventana Ultra BenchMark, Tucson, Arizona by heating at 95'C Ventana CC1 Solution at pH 8.0 for 52 min for CD10, BCL6, BCL2, and CD23 and for 36 min for MUM 1 and Ki67. Sections were then rinsed thoroughly with water and placed in a Tris-buffered saline for 5 min. All detection steps were done using the Ventana Ultra View Universal DAB KIT; all the aforementioned antibodies were incubated for 32 min at 37°C (on the instrument).

All cases were microscopically reviewed along with the IHC slides by two independent pathologists. IHCs of interest were CD10, BCL6, MUM1, BCL2, CD23, and Ki67. A cutoff of at least 30% expression by the neoplastic cells was considered positive. A cutoff of 80% Ki67 was estimated to further divide Ki67 into low and high proliferative index. A different hematologist reviewer blinded to the morphologic and immunophenotypic findings reviewed the individual patients' electronic medical record for the clinical outcomes. The treatment response evaluation was based on new Lugano classification.[12] Patients with deauville scores of 1, 2, or 3 by PET/CT or had regression of nodal mass to <1.5 cm of longest transverse diameter by CT is considered complete response (CR). All other results were considered no response (NoR) and this included patients who failed to achieve CR with the first therapy line, relapsed after first CR, developed therapy-related complications, sent to hospice, and died due to DLBCL.

Overall survival is defined as the time from DLBCL diagnosis to death. Statistical analyses were conducted using the Fisher's exact test (two tailed) and Kaplan–Meier survival curves. Significance was set at $P < 0.05$.

RESULTS

Forty-one patients with DLBCL, NOS met the inclusion criteria and were included in this study. This cohort was 44% male with a mean age of 58.2 years. Of all DLBCL cases, 59% were GCB subtype and 41% were ABC subtype. All patients were treated with curative intent with chemotherapy and/or radiation, of which 34% of patients achieved CR to therapy. The mean overall survival for all patients was 68.7 months. The description of the included patients is reported in Table 1.

Response to therapy

CD10 immunostaining was performed in all patients followed by BCL6, BCL2, and MUM1 in 32, 24, and 23 patients, respectively. The description of the results is reported in Table 2 and Figure 2. There was no association between expression of CD10, BCL6, MUM1, and BCL2 with response to therapy, 71% vs. 29%, $P = 0.51$, 55% vs. 45%, $P = 0.25$, 62 vs. 38, $P = 0.41$ and 61% vs. 39%, $P = 0.67$, respectively.

CD 23 immunostaining was reported only in four patients in our cohort. Therefore, further analysis of association of CD23 staining with therapy response and overall survival is not reported owing to lack of statistical relevance.

Figure 1: (1) GCB subtype DLBCL case that is CD10 positive, BCL6 negative, MUM1 negative with high Ki67 proliferation index of >90%. (2) ABC subtype DLBCL case that is CD10 negative, BCL6 positive, MUM1 positive with low Ki67 proliferation index o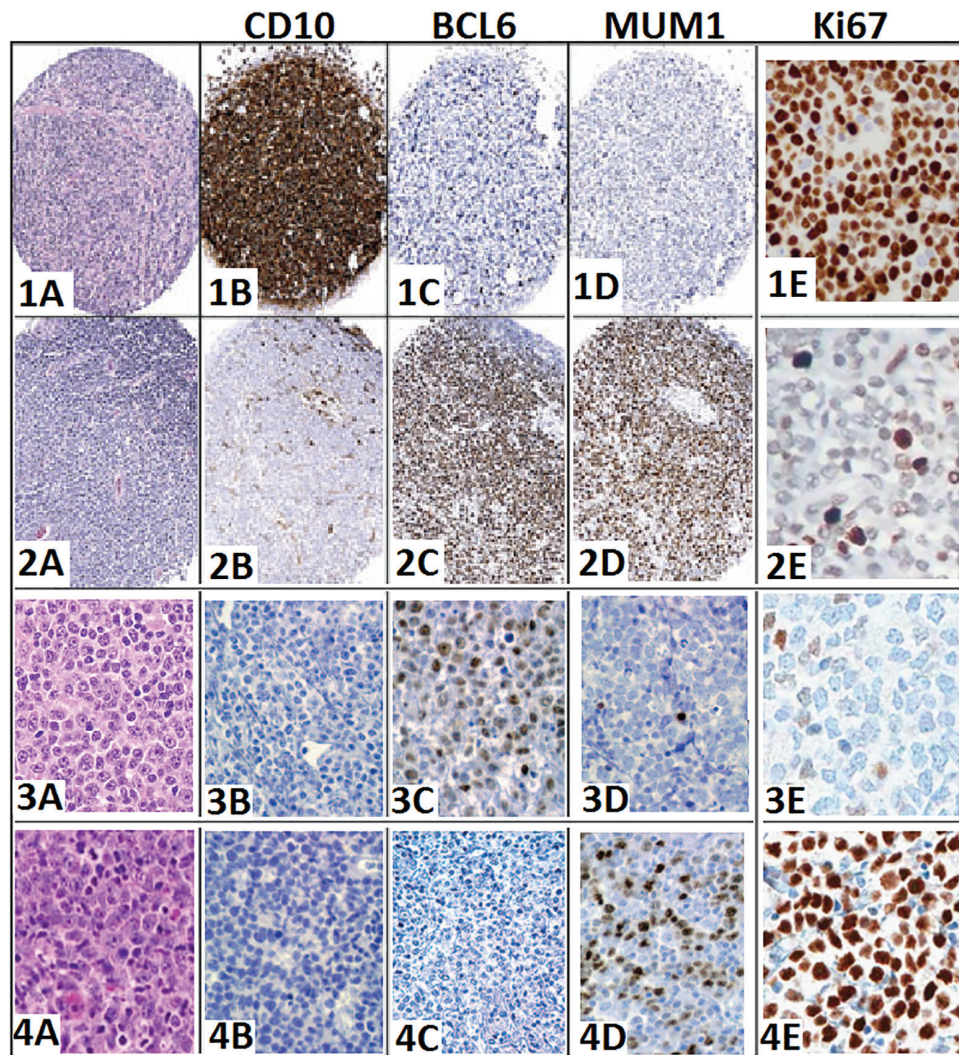f 10%. (3) GCB subtype DLBCL case that is CD10 negative, BCL6 positive, MUM1 negative with low Ki67 proliferation index of <5%. (4) ABC subtype DLBCL case that is CD10 negative, BCL6 negative, MUM1 positive with high Ki67 proliferation index of 80%

Ki67 proliferation index was reported in 38 patients. There was no association between high or low Ki67 proliferation index with NoR to therapy, Ki67 >80%: 75% vs. 25% and Ki67 <80%: 61% vs. 39%, $P = 0.48$.

Cell of origin was reported in all patients. Both GCB and ABC also showed NoR to therapy than CR (GCB: 68% vs. 32% and ABC%: 65% vs. 35%). This association was not statistically significant ($P = 1.00$).

Overall survival

The description of the results is reported in Figure 3. Using Kaplan–Meier analysis, longer mean overall survival (months) was noted with the expression of BCL6 (70.9 vs. 30, $P = 0.6$), MUM1 (78.3 vs. 63.3, $P = 0.836$), and BCL2

markers (71.3 vs. 33.1, $P = 0.384$). Shorter mean overall survival (months) was noted with the expression of CD10 (58.4 vs. 79, $P = 0.565$), high proliferative index (Ki 67) of >80% (30.7 vs. 75.9, $P = 0.002$), and GCB type rather than ABC type (58.4 vs. 79, $P = 0.565$).

Cases with Ki67 > 99%

Only three cases (7.3%) reported a Ki67 proliferation index >99%. As expected, all were of the ABC subtype and BCL2 negative showed NoR to therapy and inferior survival.

DISCUSSION

Although the GCB subtype of DLBCL predicts better outcomes compared to ABC subtype, several studies

Case no.	Age and gender	CD10 stain	BCL6 stain	MUM1 stain	BCL2 stain	CD23 stain	ki67% index (>80%)	COO subtype	Therapy response	Overall survival (months)
1	68 F	+	NA	NA	NA	NA	–	GCB	NoR	NA
2	58 M	+	NA	NA	NA	NA	–	GCB	NoR	12
3	69 M	+	NA	NA	NA	NA	+	GCB	NoR	3
4	55 M	+	+	NA	+	NA	+	GCB	NoR	12
5	20 F	+	NA	NA	NA	NA	–	GCB	NoR	124
6	45 M	+	NA	NA	NA	NA	–	GCB	NoR	20
7	49 M	+	+	NA	–	NA	+	GCB	CR	NA
8	21 F	+	NA	NA	NA	NA	–	GCB	NoR	116
9	60 M	+	NA	NA	NA	NA	NA	GCB	NoR	12
10	87 F	+	+	NA	NA	NA	–	GCB	NoR	103
11	66 M	+	+	NA	NA	NA	+	GCB	NoR	37.3
12	37 F	–	+	–	NA	NA	–	GCB	CR	99
13	37 M	+	+	NA	–	NA	+	GCB	NoR	25.3
14	54 F	–	+	+	+	NA	+	ABC	NoR	148
15	59 F	–	+	+	+	NA	–	ABC	NoR	27
16	59 F	–	+	+	+	NA	–	ABC	NoR	28
17	59 F	+	+	NA	+	NA	–	GCB	NoR	27
18	58 F	+	+	NA	+	NA	–	GCB	CR	89
19	58 M	+	+	NA	NA	NA	–	GCB	NoR	78
20	52 M	–	+	+	+	NA	–	ABC	NoR	25.6
21	65 M	–	–	NA	–	NA	–	ABC	NoR	28
22	51 M	+	+	–	+	NA	–	GCB	NoR	82.5
23	68 M	+	+	–	–	NA	–	GCB	CR	81
24	65 M	–	+	+	+	NA	–	ABC	NoR	4
25	66 M	–	+	+	+	NA	–	ABC	CR	80
26	58 F	+	+	–	+	+	–	GCB	CR	54.6
27	55 M	+	+	–	+	–	–	GCB	CR	76
28	70 F	–	+	+	–	NA	+	ABC	CR	21
29	71 F	+	+	–	NA	NA	+	GCB	CR	55
30	79 M	+	NA	NA	+	NA	–	GCB	CR	61
31	53 M	+	+	–	–	NA	+	GCB	NoR	10.3
32	58 M	+	+	–	NA	NA	+	GCB	NoR	1
33	58 F	–	+	+	+	NA	–	ABC	CR	59
34	63 F	–	+	–	+	NA	–	GCB	CR	58
35	94 F	–	–	NA	+	+	–	ABC	NoR	1
36	41 F	–	+	+	NA	+	–	ABC	CR	64
37	56 M	–	NA	+	+	NA	+	ABC	NoR	17.6
38	55 M	+	+	–	+	NA	–	GCB	NoR	17.3
39	62 M	–	+	+	NA	NA	–	ABC	CR	59.5
40	52 M	–	+	+	NA	NA	+	ABC	NoR	7
41	77 F	–	–	+	NA	NA	–	ABC	NoR	61

ABC = activated B-cell subtype, COO = cell of origin, CR = complete response, F = female, GCB = germinal center B-cell subtype, M = male, NA = not applicable, NoR = no response to therapy, + = positive, – = negative

have questioned this conclusion.[7,8] Our results showed a nonsignificant association between GCB and shorter overall survival with no conclusive results in regard to response to therapy. Recent studies assessed the clinical relevance and the prognostic value of the single marker in the Hans algorithm (CD10, MUM1, and BCL6). CD10 is a membrane-associated, neutral endopeptidase that is expressed in a variety of human tissues as well as in the germinal center cells of reactive lymphoid tissues. It is also strongly associated with the GCB-DLBCL subtype.[13] Conflicting results have been reported in the literature with a few studies showing that CD10 expression in DLBCL is associated with inferior survival,[14,15] whereas other reports show that it is associated with better prognosis.[16] However, our analysis showed both correlations were not statistically significant.

MUM1/IRF4 (Interferon Regulatory Factor-4) reflects the final steps of germinal center B-cell maturation into the plasma cell.[17] When the expression of these markers is retained in DLBCL, they are designated as postgerminal center or activated B-cell-like (ABC) subtypes. Studies have shown that MUM1 is associated with unfavorable prognosis in DLBCL.[18-22] However, our analysis showed both correlations were not statistically significant.

BCL6 is a transcription factor that prevents terminal B-cell differentiation. BCL6 is associated with the GCB subtype. BCL6 has emerged as a critical therapeutic target in DLBCL, as the first rationally designed transcription factor inhibitor.[23] Conflicting results have been reported in the literature whether BCL6 expression had no significant association,[24,25] associated with better,[26] or worse[19-21,24,27,28] overall survival in DLBCL. Our study is consistent with the former finding.

BCL2 (B-cell lymphoma 2) protein is an anti-apoptotic protein inhibiting cells from programmed cell death.[29] BCL2 gene amplification and translocations are common mechanisms causing BCL2 protein overexpression in DLBCL. Although both DLBCL subtypes express BCL2, its impact on prognosis may depend on the subtype.[30] BCL2 expression has a significant unfavorable impact on overall survival in GCB-DLBCL but not ABC-DLBCL treated with R-CHOP.[9,31] However, this result is still controversial.[9] Our cohort analysis shows the BCL2 expression is associated with NoR to therapy and longer overall survival; however, this association was not statistically significant. In addition to that, recent data showed that c-MYC/BCL2 protein coexpression in non-GCB subtype constituted a unique group with extremely inferior outcome.[32]
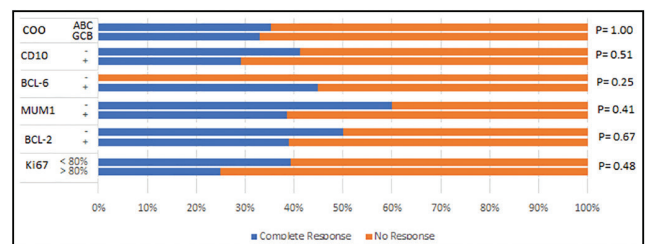

Figure 2: Fisher's exact test of the association between the immunostain expression and response to treatment in patients with DLBCL

Table 2: Fisher's exact test of the association between the immunostaining expression and response to treatment in patients with DLBCL

Immunostain	Result	CR (n = 14)	NoR (n = 27)	Total (n = 41)	Fisher's exact test (P Value)
CD10 (n = 41)	Positive	29%	71%	24	0.51
	Negative	41%	59%	17	
	Total reported	14	27	41	
BCL6 (n = 32)	Positive	45%	55%	29	0.25
	Negative	0%	100%	3	
	Total reported	13	19	32	
MUM1 (n = 23)	Positive	38%	62%	13	0.41
	Negative	60%	40%	10	
	Total reported	11	12	23	
BCL2 (n = 24)	Positive	39%	61%	18	0.67
	Negative	50%	50%	6	
	Total reported	10	14	24	
ki67 (n = 40)	> 80%	25%	75%	12	0.48
	< 80%	39%	61%	28	
	Total reported	14	26	40	
COO (n = 41)	GCB	32%	68%	24	1.00
	ABC	35%	65%	17	
	Total reported	14	27	41	

ABC = activated B-cell subtype, CR = complete response, GCB = germinal center B-cell subtype, NoR = no response to therapy
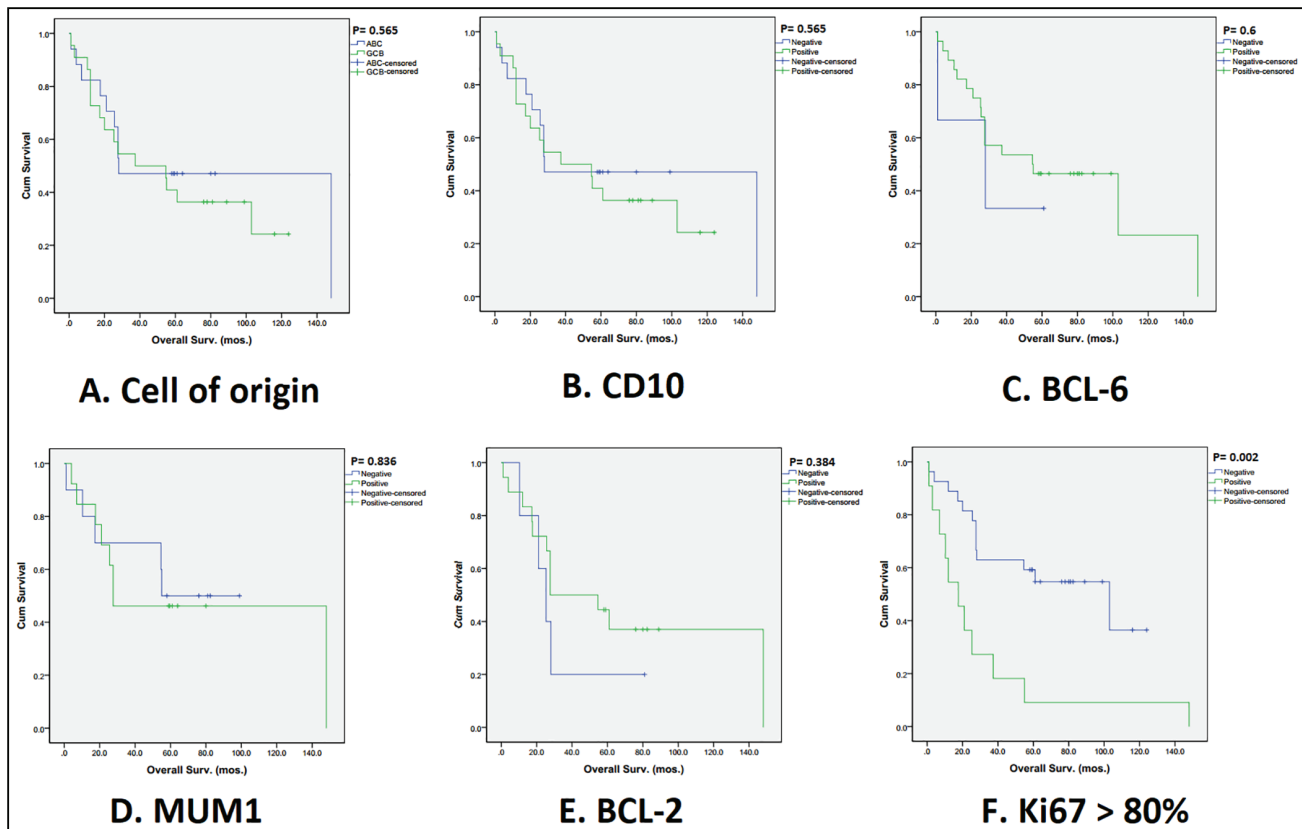
Figure 3: Kaplan–Meier survival analysis in patients with DLBCL

CD23, the FC fragment of the IgE receptor, is a surface marker present on follicular dendritic cells and naïve B cells. In lymphoid neoplasms, CD23 is routinely expressed in chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) cases and most mediastinal large B-cell lymphoma cases. CD23 is only present in approximately 16% of DLBCL cases.[33] Its prognostic value is not well established[33] but has been associated with favorable outcome and better overall survival in several studies.[10,30,33] Our cohort showed the CD 23 immunostaining is only reported in four patients, which preclude further analysis of clinical association.

Ki67 is a nuclear nonhistone protein that is expressed in all phases of the cell cycle except the resting stage (G0). Ki67 has been used in clinical practice as an index to evaluate the proliferative activity of lymphoma with controversial results of its association with DLBCL subtypes.[34,35] High Ki67 expression is a predictive factor of unfavorable survival in DLBCL patients treated with chemotherapy.[11,36-38] Our results showed that high Ki67 proliferation index (>80%) showed a statistically significant association with shorter overall survival and nonsignificant association with unfavorable response to treatment. However, other studies showed controversial results of no clinical relevance of Ki67 expression in DLBCL.[39,40] Moreover, the Ki67 expression is thought to be a new unfavorable prognostic factor in

DLBCL patients with bone marrow involvement treated with R-CHOP.[41] We believe the controversial results are based, in part, on the different threshold values used to define high vs. low Ki67 expression status. Spyratos *et al.* suggested that the Ki67 cutoff should be chosen according to the clinical objectives. If Ki67 is used to exclude patients with slowly proliferating tumors after chemotherapy treatment, a low cutoff will help to avoid overtreatment. On the contrary, if Ki67 is used to identify patients sensitive to chemotherapy treatment, a high cutoff is preferable.[36,42] Moreover, Ki67 has significant interobserver variability between interpreting pathologists, which may also contribute to the conflicting results between studies.

Limitations

This study is limited by the small number of patients. Also, CD23 immunostain was only reported in four patients so further analysis of association of CD23 with therapy response and overall survival is not reported. Another limitation is the unavailability of molecular gene expression profile (GEF) in our data. The GEP using microarrays has been used to subtype DLBCL into GCB vs. ABC. The microarray analysis reported consistent results with IHC that patients with DLBCL expressing a GEP of GCB have a longer survival than those with a GEP of ABC.[43] However, its complexity and cost make it impracticality of perform

microarray analysis on every patient with DLBCL and thus the immunohistochemical algorithms are still the most common method. Nevertheless, the strengths of this report are derived from the minimum 5-year follow-up of the patients.

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Conflicts of interest
There are no conflicts of interest.

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