

RESEARCH ARTICLE

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Molecular Subtypes, Apoptosis and Proliferation Status in Indonesian Diffuse Large B-Cell Lymphoma Cases

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Abstract

Objective: The diffuse large B-cell lymphoma (DLBCL) has two major molecular subtypes, germinal center B-cell-like (GCB) and non-GCB. These have differing behavior which affects overall patient survival. However, immunohistochemistry based molecular subtyping of Indonesian DLBCLs has been limited. This was the focus of the present study, with a focus of attention on the apoptotic index (AI) and the proliferation index (PI) of the two molecular subtypes. **Materials and Methods:** During the study period of 3.5 years, a total of 98 cases of DLBCL were identified. Molecular subtypes and PI were determined by immunohistochemistry and TUNEL method was used to determine the AI. **Result:** GCB accounted for 31 cases (31.6%) and non-GCB the remainder (68.4%). Gender showed a slight male predominance (54 cases, 55.1%), with a higher incidence in the extra-nodal region (57 cases, 58.2%). The AI and PI were significantly higher in GCB ($p < 0.001$ in the Mann-Whitney test) and a Spearman correlation coefficient test showed that PI was positively correlated with AI ($r = 0.673$, $p < 0.001$). **Conclusion:** The findings indicate that the non-GCB subtype is more common than GCB in Indonesian DLBCL. GCB features significantly higher PI and AI, which themselves appear linked.

Keywords: GCB- non-GCB- proliferation index- apoptotic index- DLBCL

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most frequent Non-Hodgkin's B-cell lymphoma reported worldwide (Türk et al., 2011; Hassan et al., 2012). It is characterized by heterogeneous clinical presentations, morphology, molecular and genetic features (Sandoval-Sus, Chavez and Dalia, 2016). Moreover, DLBCL represents a heterogeneous group of neoplasms rather than a single clinicopathologic entity (Hans et al., 2004). There are two molecularly distinct forms of DLBCL which are characterized by gene expression patterns indicative of different stages of B-cell differentiation, GCB (Germinal Center B-cell-like DLBCL) and non-GCB/ABC (Non-Germinal Center B-cell-like DLBCL or Activated B-cell-like DLBCL) (Blenk et al., 2007; Lenz et al., 2008; Younes, 2015; Chaganti et al., 2016). Patients with GCB have a significantly better overall survival than those with non-GCB (Reber et al., 2013; Bellas et al., 2014; Lenz, 2015; Nowakowski and Czuczman, 2015)

As gene expression profiling (GEP) is not a routinely performed diagnostic work-up, efforts have been made to find a substitute (Coutinho et al., 2013; Dobashi, 2016;

Bobée et al., 2017; Campo, 2017; Yoon et al., 2017). The Hans algorithm was the first established algorithm reported to have high sensitivity for GEP classification (Bai et al., 2005). Various algorithms with immunohistochemistry based approaches for molecular classification in DLBCL were then developed and claimed to be 80-90% accurate (Visco and Xu-Monette, 2012; Coutinho et al., 2013). In the last few years, there has been accumulating molecular and immunohistochemical evidence which indicate a link between B-cell differentiation gene expression profiles and the expression of apoptotic and cell cycle-associated genes in DLBCL (Bai et al., 2003, 2005; Bai et al., 2004). Mostly, those studies were held in Western populations and the results were very similar. The frequency of GCB was higher than non-GCB. In addition, GCB showed higher proliferation and apoptotic indices compared to non-GCB (Bai et al., 2003, 2005; Bai, et al., 2004; Hans et al., 2004; Miles et al., 2008; Al-Humood et al., 2011). Other studies in Pakistan, Kuwait, Turkey showed that non-GCB constituted more than half of DLBCL cases (Al-Humood et al., 2011; Türk et al., 2011; Hassan et al., 2012). This study was similar to a previous study in China comparing Chinese and Western populations (Chen et al., 2010). Moreover, the study showed that the frequency of

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non-GCB was higher in Chinese populations (Chen et al., 2010). Several reports have investigated proliferation and apoptotic indices regarding these molecular subtypes of DLBCL. Most studies indicate that GCB correlates with higher proliferation and apoptotic indices (Bai et al., 2003, 2005; Bai et al., 2004; Broyde et al., 2009; Chen et al., 2010; Türk et al., 2011).

Sardjito Hospital, Special Region of Yogyakarta, is a referral hospital for patients all over Indonesia and DLBCL cases are regularly found in this population. However, studies and data related to molecular subtypes, apoptosis and proliferation status of DLBCL are considerably limited. This study focused on investigating the molecular subtypes of Indonesian DLBCL and the expression profiles of apoptosis index (AI) and proliferation index (PI) in both subtypes of DLBCL.

Materials and Methods

Study Subjects

A total of 98 cases of de novo DLBCLs NOS that were diagnosed according to the 2008 World Health Organization (WHO) classification (Swerdlow et al., 2008) from January 2012 to August 2015 were obtained from the Department of Anatomical Pathology, Sardjito Hospital, Universitas Gadjah Mada, Special Region of Yogyakarta, Indonesia.

Clinicopathological parameters for all patients were obtained from patient's pathology record. Ethical committee approval from Faculty of Medicine, Universitas Gadjah Mada was also obtained for this study.

Immunohistochemistry analysis (Hans Algorithm and Ki67).

For immunostaining, 4- μ m thick sections were cut from formalin-fixed paraffin-embedded (FFPE) tissue blocks and placed on electrostatic-charged, poly-L-lysine-coated slides (Biogear, Microscope Slide). Sections were dehydrated at 45°C overnight. All immunostaining procedures including deparaffinization were performed on Semi-automatic Intellipath FLX, Biocare Medical with open kit. The antigen retrieval process was performed on Decklocking Chamber, Biocare Medical. After counterstaining all the slides with hematoxylin in semi-automated slide stainer, Leica, dehydration, incubation in xylene and the mounting processes then manually performed. After all, immunostaining procedures were completed.

The Hans algorithm of DLBCL subtypes was applied with three antibodies for CD10, BCL6 and MUM1. The proliferation index was determined by Ki67. The following primary antibodies were used in this study: CD10 (Ready-to Use, clone: 56C6, Dako AS Plus), BCL6 (dilution: 1/10, clone: PG-B6p, Dako AS, Glostrup, Denmark), MUM1 (dilution: 1/50, clone: MUM1p, Dako SA, Glostrup, Denmark) and Ki67 (Ready-to-Use, clone: MIB-1, Dako SA, Glostrup, Denmark). Reactive lymph nodes tissue samples were used as positive controls. Negative controls were treated with the same immunohistochemical method by omitting the primary antibody.

Immunohistochemistry was interpreted as positive when the following staining patterns occurred with respective antibodies: CD10 (cell membrane and cytoplasmic staining), BCL6, MUM1 and Ki67 (nuclear staining). Ten fields selected on the basis that they contained immunopositive cells were counted by 40x objective lens on the light microscope. The number of immunopositive cells was divided by the total number of the counted cells and the expression was defined as the percentage of positive cells. The cut-off level for interpreting CD10, Bcl-6 and MUM1 as positive was >30% tumor cell staining (Coutinho et al., 2013). Based on The Hans Algorithm, specimens that were CD10+ were categorized as GCB. For CD10- specimens, the procedure was continued with BCL6. CD10-/BCL6- specimens were categorized as Non-GCB. CD10-/BCL6+ specimens were used for staining with antibody against MUM1. Moreover, CD10-/BCL6+/MUM1+ specimens were determined as non-GCB, while CD10-/BCL6+/MUM1- specimens were determined as GCB. We also divided the Ki67 index into 2 groups: low Ki67 (\leq 70%) and high Ki67 (>70%) (Broyde et al., 2009).

TUNEL assay

The terminal deoxynucleotidyl-transferase (TdT)-mediated in situ labeling technique (TUNEL; Peroxidase in situ Apoptosis Detection Kit, ApopTag, Merck Millipore) was performed on the 4- μ m thick sections of formalin-fixed paraffin-embedded tissue for demonstration of DNA fragmentation. Positive and negative controls were included in each staining. Apoptosis within the germinal centers of reactive lymph nodes served as positive controls. Negative controls consisted in the same method with omission of the TdT reaction step. After deparaffinization and dehydration, slides were rinse in distilled water. Next, tissue sections were digested by incubation for 15 minutes with proteinase-K at room temperature, then rinsed in phosphate-buffered-saline (PBS). Peroxidase activity was blocked by incubation for 5 minutes in 30% hydrogen peroxide in PBS at room temperature. After application of an equilibrium buffer for 5 minutes the slides were incubated with working strength TdT enzymes and reaction buffer for 1 hour at 37°C in the dark. The reaction was stopped by prewarmed (37°C) working strength stop/wash buffer then rinsed in PBS. The incorporated nucleotides were identified by adding anti-digoxigenin, incubated for 1 hour at 37°C then rinsed in PBS. Afterwards, slides were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB substrate, Roche) for 10 minutes at room temperature, counterstained with Mayer' hematoxylin and mounted.

According to Bai et al., morphologically intact TUNEL-positive cells and apoptotic cells in hematoxylin-eosin stained slides (defined as cells with condensed, hyperchromatic, ring-like, crescentic, or beaded chromatin and often surrounded by a clear halo) were considered positive and are referred to as apoptotic cells (Bai et al., 2004). Areas of obvious necrosis were excluded from counting. The number of apoptotic cells was recorded by using the 40 \times objective lens and by counting the apoptotic cells in at least 10 randomly selected fields, corresponding

to a total of 2,000 to 3,000 cells. The apoptotic index was determined as the number of apoptotic cells expressed as a percentage of the total number of counted cells.

Statistical Analysis

X² test, the Mann-Whitney test and the Spearman correlation coefficient test were applied for statistical analysis. The results were considered statistically significant when P<0.05. Statistical analyses were performed using IBM SPSS Statistics 22.0 program for Windows.

Results

Patients Characteristics

Samples from 98 Indonesian DLBCL, NOS were included in this study. Gender was distributed as 54 males (55.1%) and 44 females (44.9%). The median age of the patients was 56.50 years (range, 2-83 years). Fifty-five patients (56.1%) were 60 years or under and 43 patients (43.9%) were over 60 years. Based on the site, 41 cases (41.8%) were nodal disease and 57 (58.2%) were extra-nodal disease at initial diagnosis (Table. 1).

Molecular subtypes, Apoptosis and Proliferation Status

Immunohistochemical expressions of CD10, BCL6 and MUM1 are shown in Figure. 1, A-F and details summarized in Table 2. Some cases were only stained with CD10 and not BCL6 and MUM1. The mean PI was 42.30% (±31.83) as assessed by Ki67 staining (Figure 2, A-B). The mean AI was 14.036% (±8.55) as determined by the TUNEL method (Figure 2C). Two molecular subtypes were distinguished based on the pattern of differentiation described by the Hans algorithm: a) GCB

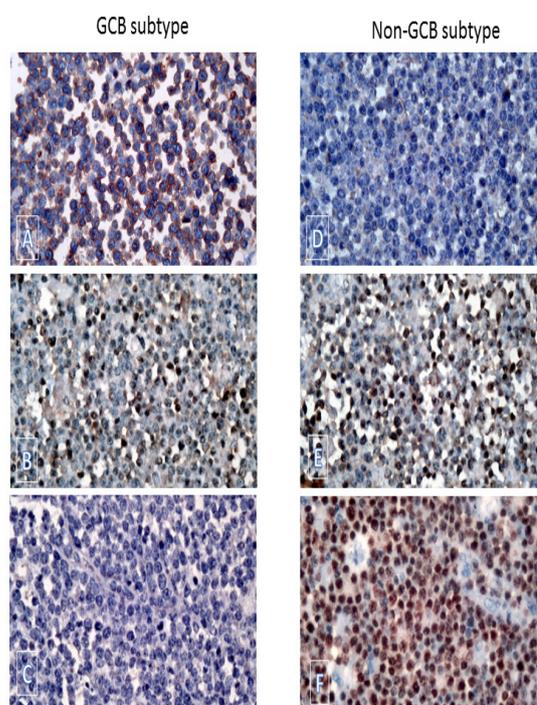


Figure 1. Immunohistochemical Expression of GCB Subtype with CD10+ (a), bcl-6+ (b), MUM1- (c) and Non-GCB Subtype with CD10- (d), bcl6+ (e), MUM1+ (f). (x200)

profile: 31/98 (31.6%) cases (CD10+: 30 cases, BCL6+/MUM1-: 1 cases) and b) non-GCB profile: 67/98 (68.4%) cases, all showed CD10- with some variations (CD10-/BCL6- and CD10-/BCL6+/MUM1+) (Table. 2). The results concerning GCB and non-GCB subtypes associated with age (p= 0.294), sex (p= 0.402) and site (p= 0.182)

Table 1. Clinical Features of Indonesian Diffuse Large B- Cell Lymphoma Patients (Total n=98)

Clinical features	GCB n (%)	Non-GCB n (%)	P values	High PI n (%)	Low PI n (%)	P values
Age(y)						
Median	56.5					
Range	2-83					
≤60	55	15 (27.3)	40 (72.7)	0.294	11 (20.0%)	44 (80%)
>60	43	16 (37.2)	27 (62.8)		15 (34.9%)	28 (65.1%)
Sex						
Male	54	19 (35.2)	35 (64.8)	0.402	15 (27.8%)	39 (72.2%)
Female	44	12 (27.3)	32 (72.7)		11 (25.0%)	33 (75.0%)
Site						
Nodal	41	16 (39.0)	25 (61.0)	0.182	13 (31.7%)	28 (68.3%)
Extra-nodal	57	15 (31.6)	42 (73.7)		13 (31.7%)	44 (77.2%)

GCB, Germinal Center B-cell-like; non-GCB, non Germinal Center B-cell-like; PI, Proliferation Index.

Table 2. CD10/BCL6/MUM1 Molecular Subtypes (Total n=98)

	CD10			BCL6				MUM1			
	(+)	(-)	Σ	(+)	(-)	N/A (n)	Σ	(+)	(-)	N/A (n)	Σ
	(n)	(n)	(n)	(n)	(n)	(n)	(n)	(n)	(n)	(n)	(n)
GCB	30	1	31	5	0	26	31	4	9	18	31
Non-GCB	0	67	67	4	63	0	67	21	2	44	67

(+), positive expression; (-), negative expression; Σ, Total; n: number of cases; N/A, not applicable/not performed

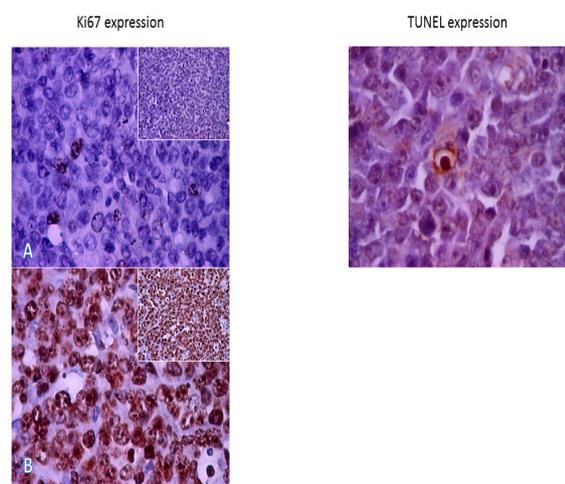


Figure 2. Immunohistochemical Expression of Ki67 Showed Low (A) and High (B) Proliferation Index (x400, inset x200). Staining of apoptotic cells by the TUNEL method (x400)

Table 3. GCB, Non-GCB Associated with High and Low PI Using Chi- Square Test (Total n=98)

	High Proliferation Index		Low Proliferation Index		P value
	n	%	n	%	
GCB	17/31	54.8	14/31	45.2	<0.001*
Non-GCB	9/67	13.4	58/67	86.6	

*, indicates the statistically significant correlation ($p < 0.05$)

were not significant (Table 1). Chi-square test results were performed to analyze the PI association to age ($p = 0.098$), sex ($p = 0.757$) and site ($p = 0.325$) but were not significant (Table 1). PI associated with molecular subtypes showed significant results ($p < 0.001$). Non-GCB showed 58/67 (86.6%) cases with low proliferation index while GCB showed 17/31 (54.8%) cases with high proliferation index (Table 3).

The Mann-Whitney test was used to analyze GCB and non-GCB subtypes in relation to AI and PI. The GCB subtype was significantly associated with a higher PI and AI (both showed $p < 0.001$) (Table 4). The correlations of PI and AI was analyzed using the Spearman correlation coefficient test which showed that expression of PI was positively correlated with AI ($r = -0.673$, $p < 0.001$).

Discussion

Many algorithms based on immunohistochemistry approached have been established to distinguish the molecular subtypes of DLBCL (Jack et al., 2005; Bajwa et al., 2017). The Hans algorithm is the most widely used, and it performed well in this study. It showed that the frequency of non-GCB is higher than GCB in Indonesian DLBCL. The present study demonstrated similar results compared to the other studies in Asian countries such as Korea, China, and Japan (Chen, 2006; Oh and Park, 2006; Shiozawa et al., 2007). Some studies investigated the reason that the proportion of non-GCB in Asia is higher than in western countries. Based on the site, several studies

Table 4. The Molecular Subtypes in Relation to the PI and AI Using Mann- Whitney Test

	Mean of positive expression (%)	Molecular subtype	Mean rank	P values
PI	68.06 (± 23.618)	GCB	72.26	<0.001*
	30.37 (± 27.913)	Non-GCB	38.97	
AI	22.47 (± 8.359)	GCB	80.18	<0.001*
	10.13 (± 5.188)	Non-GCB	35.31	

*, indicates the statistically significant correlation ($p < 0.05$); PI, Proliferation Index; AI, Apoptotic Index

have shown that the majority of extra-nodal DLBCL have the non-GCB phenotype, suggesting that the proportion of GCB and non-GCB subtypes in DLBCL may be affected by the difference in proportion of nodal and extra-nodal cases (Tai and Peh, 2004). Similarly, the majority of cases in our study presented with extra-nodal disease and a higher percentage of cases had the non-GCB profile. However, Chi-square analysis determined an insignificant relationship exists between molecular subtypes and site, also with age and sex. Earlier studies observing nodal and extra-nodal DLBCL reported that genomic differences appear to be genuinely related to the site of disease and not the molecular subtypes. Nodal DLBCLs have an elevated expression level of BCL2 protein, which seemingly arise from increased copy number/amplification at 18q chromosome arm (Al-Humood et al., 2011). Another study reported that BCL2 protein is frequently expressed in low grade MALT lymphoma but is lost in high grade tumors (de novo extra-nodal DLBCL) (Hashmi et al., 2014).

PI is an important prognostic and predictive parameter in DLBCL for determining response to chemotherapy (Yoo and Kim, 2009; Abdelhamid et al., 2011; Hashmi et al., 2014). Many studies reported this with different cut-off values of Ki67 (Bai et al., 2004; Al-Humood et al., 2011; Dunleavy and Wilson, 2014; Payandeh, 2015; Zeggai et al., 2016; Tang et al., 2017). Broyde et al., (2009) set a cut-off value of 70% as favorable and poor prognosis. Using the same cut-off value, we defined PI as low and high PI. Most cases showed low PI in relation to age, sex and site, but the results were not significant. Instead, these findings showed an opposite result ($p < 0.001$) in relation to molecular subtypes. PI was found to be low in 86.6% of non-GCB cases and high in 54.6% GCB cases.

The Mann-Whitney test showed significant relationships between molecular subtypes of DLBCL, AI and PI. GCB subtype showed higher expression of both AI and PI than non-GCB. In relation to that pattern, CD10 and BCL6 are two major proteins expressed in GCB. Data in the literature demonstrates that CD10 and BCL6 can either promote or inhibit apoptosis or cell cycle regulation depend on the cellular context and experimental approach used (Bai et al., 2003).

In this study, increased AI and PI indicated both CD10 and BCL6 act to promote proliferation and apoptosis. Increased AI and PI in DLBCL were associated with increased expression of CD10 and BCL6 (Cutrona et al., 1999; Bai et al., 2003; Bai et al., 2004; Hans et al., 2004; Miles et al., 2008; Al-Humood et al., 2011).

The association between BCL6 and PI might be due,

in part, to the possibility that BCL6 confers resistance to anti-proliferative signals from the p19 (ARF)-p53 pathway, downregulates the expression of the CDKI p27 and blocks Blimp-1 expression. Decreased p27 expression in combination with altered p53/Rb/p16 expression status is significantly associated with enhanced proliferation in DLBCL (Bai et al., 2004). PRDM1/Blimp-1, a gene which expression is required for terminal differentiation to plasma cells, is a direct target of BCL6. Terminal differentiation defines the process by which a GC B-cells becomes a plasma cell and it involves a change in antibody production and is associated with a loss of proliferative capacity. The unifying concept is that high BCL6 expression is associated with relatively high proliferative capacity whereas low BCL6 and high PRDM1 are associated with differentiation to effector cells (Wagner, 2010; Testoni et al., 2015). BCL6 may also promote cell proliferation directly by repression of expression of the cell cycle inhibitor p21. It also inhibits DNA repair pathways and TP53. Perturbation of both these pathways may contribute to normal GC B-cell function by repressing DNA damage responses and permitting somatic hypermutation, but in the context of malignancy, this could lead to mutations promoting aggressive lymphomas (Ohno, 2004; Wagner and Ferrigno, 2010).

In relation to the higher expression of the AI in GCB than non-GCB, overexpression of BCL6 may induced apoptosis via downregulation of the anti-apoptotic genes BCL2 and BCLx1 (Bai et al., 2003; Miles et al., 2008).

BCL6 rearrangements were associated with good clinical outcome and GCB profile which had BCL6 mRNA as a characteristic marker was also associated with improved outcome to treatment with CHOP regimen (Bai et al., 2003; Bai et al., 2004; Ohno, 2004; Tai and Peh, 2004; Wagner, 2010; Dunleavy and Wilson, 2014).

In addition to BCL6, CD10 expression was also positively correlated with the AI and PI. There are several evidences indicating those relationships, such as: (1) GC cells are characterized by high proliferation and have the propensity to undergo apoptosis and upregulate CD10 expression on apoptotic induction, whereas CD10 is absent on other subsets of mature B-cells that are not characterized by high apoptosis. (2) Burkitt lymphoma's cells that are characterized by high proliferation and apoptosis almost constantly express CD10 and (3) CD10-positive B-acute lymphoblastic leukemia cells were cycling cells with elevated c-myc and propensity to apoptosis, whereas CD10-negative B-acute lymphoblastic leukemia cells had lower cycling capacities and c-myc levels and were resistant to apoptosis. Moreover, human post-thymic and thymic T-cells express CD10 when undergoing apoptosis (Bai et al., 2003, 2005, 2007; Türk et al., 2011). (Bai et al., 2003, 2005, 2007; Türk et al., 2011)

To explain the relations between CD10 and apoptosis it was suggested that CD10 might degrade cytokines that reach the cell when apoptosis has already started (Bai et al., 2003). Because a variety of cytokines may play a protective role in B- and T-cell apoptosis, CD10 expression may potentiate the apoptotic ability of B and T cells, by inhibiting the protective signals. This could be consistent with the capacity of CD10 to hydrolyze

a variety of active peptides, including growth and chemotactic factors (Cutrona et al., 1999). It is possible that CD10 participates in the process of selection in the germinal center and the thymus by increasing the threshold of cytokines required to prevent B and T-cell apoptosis, respectively (Shaffer et al., 2000).

In addition, the PI showed a significant positive correlation with the AI with Spearman correlation coefficient test. This result provides further support to the accumulating evidence that apoptotic and proliferation indices are positively correlated in DLBCL and the expression of the GCB-cell-associated BCL6 and CD10 proteins is associated with increased proliferation and apoptosis status (Hans et al., 2004; Miles et al., 2008; Al-Humood et al., 2011, Bai et al., 2004).

In conclusion, the present study shows: the Non-GCB profile in Indonesian DLBCL is higher than GCB, and the GCB profile is associated with higher expression of AI and PI. This may be a result of high CD10 and BCL6 expression in the GCB profile. Therefore, patients with the GCB profile may be more sensitive to treatment compared to non-GCB patients. Moreover, these findings may explain the aggressive behavior and unfavorable effects observed in the clinical outcome of Indonesian DLBCL patients who our clinicians observe on a daily basis. Genetic signatures for nodal and extra-nodal DLBCL are also considered important to identify since they may have diagnostic and prognostic implications in DLBCL cases.

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