

Supplement Article

IX. Is it only about MYC? How to approach the diagnosis of diffuse large B-cell lymphomas

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma (NHL), representing around 30% to 40% of all newly diagnosed lymphomas [1]. DLBCL is clinically, morphologically and biologically a heterogeneous disease reflected in the highly variable clinical course. The 2008 World Health Organization (WHO) classification of lymphoid malignancies recognizes within the group of DLBCL, not otherwise specified (NOS) several subtypes characterized by unique clinical and pathological features including primary DLBCL of the central nervous system (CNS), primary cutaneous DLBCL, leg type, T-cell histiocyte-rich large cell lymphoma and EBV positive DLBCL of the elderly (Table 1). Nevertheless, most cases of DLBCL fall into the 'NOS' category. In the last 15 years our understanding of the genetic changes and biology of DLBCL has increased tremendously [2]. Gene expression profiling (GEP) studies have revealed that DLBCL comprises several molecular subgroups that reflect either the stage in B cell development from which the disease originates or the activity of different biological programs [3,4]. The standard initial treatment for DLBCL is combined immunochemotherapy with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) [5]. While durable remissions can be achieved in the majority of cases with this combined regimen, over 30% of patients will not respond or will relapse with resistant disease. A possible explanation for such differences in therapeutic success is the considerable biological heterogeneity of DLBCL. Therefore, there is an ongoing effort to tailor therapy based on specific subtypes of DLBCL, and to identify prognostic markers like *BCL2* and *MYC*. The diagnosis of DLBCL needs to integrate, in addition to classic morphology and immunophenotype, all the new genetic and molecular diagnostic tools. This report attempts to review

distinctive pathological characteristics of DLBCL and their clinical significance.

Molecular features

DLBCL is characterized by monoclonal rearrangement of immunoglobulin heavy (*IGH*) and light chain (*IGκ-IGλ*) genes. Analogous to most B-NHL, DLBCL derives from a mature B cell that has experienced the germinal center (GC) reaction. Based on GEP studies DLBCLs have been divided into two main subgroups based on the putative cells of origin [3,4]. GC B cell-like (GCB)-DLBCL (50% cases) exhibits a transcriptional profile that resembles that of GC B-cell with expression of genes normally detected in GC B-cells such as CD10 and the transcriptional repressor *BCL6* and harboring highly mutated immunoglobulin genes with ongoing somatic hypermutations (SHM). Activated B cell-like (ABC)-DLBCL shows several features of B cell receptor (BCR) activated B-cells at a plasmablastic stage, just prior to exit the GC with up-regulation of genes required for plasma cell differentiation (IRF4/MUM1) (Figure 1). These tumors downregulate the GC-specific program, activating at the same time, the NF-κB and BCR signaling pathways [2]. Consistent with their late GC origin, these tumors do not show evidence of ongoing SHM. Because not all cases of DLBCL can be characterized as GCB or ABC, a less well-characterized group comprising about 15% of the cases remain unclassifiable.

Genetic alterations

Within the group of mature B cell NHL, DLBCL shows the highest degree of genomic complexity including point mutations and copy number aberrations, and less frequently chromosomal translocations and gene amplification [2]. Although some of these lesions might be observed

Table 1. Diffuse large B-cell lymphomas in the 2008 WHO Classification

Diffuse large B-cell lymphoma, not otherwise specified
Primary DLBCL of the CNS
Primary cutaneous DLBCL, leg type
T cell/histiocyte rich large B-cell lymphoma
EBV+ DLBCL of the elderly*
Diffuse large B-cell lymphoma associated with chronic inflammation
Primary mediastinal (Thymic) large B-cell lymphoma
Intravascular large B-cell lymphomas
ALK-positive large B-cell lymphoma
Plasmablastic lymphoma
Primary effusion lymphoma
Large B-cell lymphoma arising in HHV-8-associated-multicentric Castlemans disease
Burkitt lymphoma
Borderline cases§
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Hodgkin lymphoma

*Provisional entity
§Provisional categories

in both GCB and ABC subtypes of DLBCL, most of them are preferentially associated with one or the other subtype of DLBCL, suggesting their potential role for diagnostic, prognostic and therapeutic stratification (Figure 2). The frequent mutations found in DLBCL are thought to be the result of an aberrant function of the physiologic SHM

mechanism. Accordingly, mutations in *CREBBP/EP300* and *MLL2* are preferentially seen in GCB-subtype. *EZH2* is required for GC formation and mutations in this gene are restricted to GCB-DLBCL. Translocations resulting in deregulated *BCL2* and *MYC* are almost restricted to GCB-DLBCL. Around 30–40% of GCB-DLBCL carry the t(14;18) translocation, whereas *MYC* translocations have been identified in 5–15% of DLBCL [6]. In contrast, translocations involving the *BCL6* locus are present in 35% of DLBCL cases, being more frequent in ABC-DLBCL (25%). Another major transformation mechanism that impairs plasma cell differentiation are mutations and deletions in *PRDM1/BLIMP1* identified in 25% of ABC-DLBCL.

Constitutive activation of the NF-κB transcription factor complex represents the hallmark of ABC-DLBCL [2]. The underlying causes of NF-κB signaling pathway activation are diverse and include gain-of-function mutations in signal transduction components of the BCR (*CD79a* and *CARD11*) and toll-like receptor (*MYD88*) signaling pathways. Loss-of-function mutation and/or deletions in the NF-κB negative regulator *TNFAIP3/A20* have been reported in up to 30% of ABC-DLBCL.

Morphologic features

The diagnosis of DLBCL is usually not difficult. Lymph nodes demonstrate a diffuse proliferation of large lymphoid

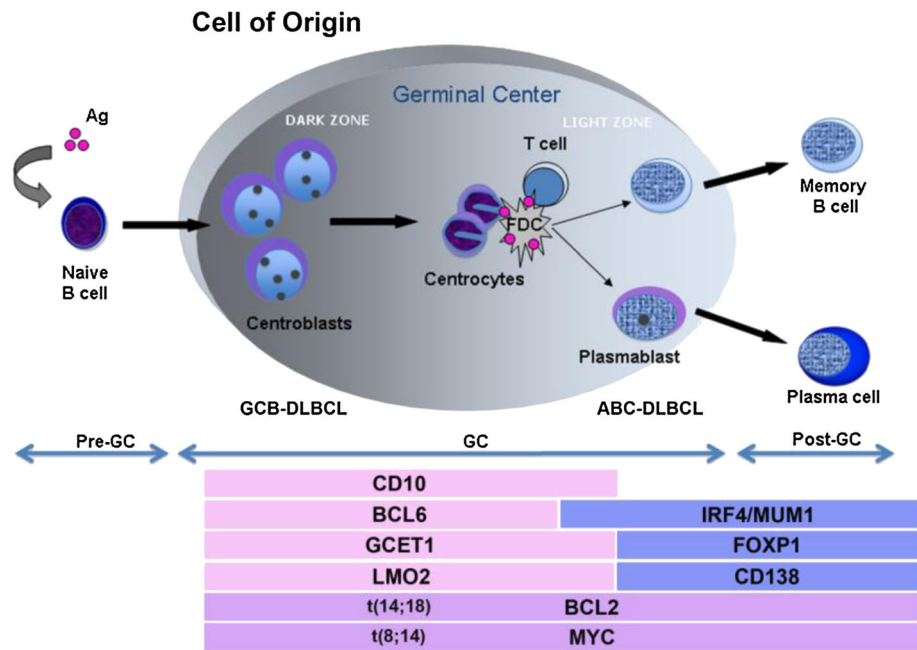


Figure 1. Determining cell of origin in diffuse large B cell lymphoma. DLBCL derives from a mature B cell that has experienced the germinal center (GC) reaction. The GCB-DLBCL derives from centroblasts whereas the ABC-DLBCL from plasmablastic cells, just prior to exit the GC. Antibodies used for GCB-type are depicted in pink, for ABC-type in blue. Prognostic markers BCL2 and MYC are depicted in purple. Ag, Antigen; GC, Germinal Center; GCB-DLBCL, germinal center B-cell diffuse large B cell lymphoma; ABC, activated B cell; FDC, follicular dendritic cell

Genes altered in both GCB- and ABC-DLBCL	
<i>Histone chromatin modifiers</i>	
	CREBBP/EP300 mutations and deletions (32%)
	MLL2/MLL3 mutations (32-38%)
	BCL6 translocations (20-40%)
	FOXO1 mutations (8%)
	TP53 mutations and deletions (20%)
Genes altered in GCB-DLBCL	
<i>Proliferation / Apoptosis</i>	
	BCL2 translocation and mutations (34-45%)
	MYC translocation and mutations (5-15%)
	miR-17-92 chromosome number gains (6-12.5%)
<i>Signaling</i>	
	TNFRSF14 mutations (13%)
	GNA13 mutations (11%)
	SGK1 mutations (13%)
	PTEN deletions (6-11%)
<i>Other</i>	
	EZH2 mutations (22%)
	BCL6 mutations (15%)
Genes altered in ABC-DLBCL	
<i>Constitutive NF-κB/BCR activity</i>	
	TNFAIP3/A20 mutations and deletions (30%)
	MYD88 mutations (30-37%)
	CD79A/B mutations (21%)
	CARD11 mutations (10%)
<i>Terminal differentiation block</i>	
	PRDM1/BLIMP1 mutations and deletions (25%)
	BCL6 translocations (25%)
<i>Apoptosis</i>	
	BCL2 amplifications (13%)
<i>Cell cycle</i>	
	CDKN2A/B deletions (24-30%)

Figure 2. Genetic alterations in DLBCL. The oncogenic pathways and the genes affected in the GCB and/or ABC subtypes of DLBCL are listed. Note that some genetic alterations are preferentially found in one subtype or the other

cells that have totally or partially effaced architecture. Cytological, DLBCL can be centroblastic, immunoblastic or anaplastic in appearance [1]. Centroblastic morphology is the most common variant composed of medium to large cells with round to oval vesicular nuclei and fine chromatin with two to four nuclear membrane-bound nucleoli. There is moderate amphophilic to basophilic cytoplasm. In the immunoblastic variant greater than 90% of the cells have an immunoblastic appearance with a large centrally located nucleolus and basophilic or amphophilic cytoplasm. This variant has been associated with the non-germinal center type derivation, an adverse prognosis [4] and recently as a major reservoir for *MYC-IGH* translocations. In most cases, however, the tumors show an admixture of centroblasts and immunoblasts. The distinction of the immunoblastic variant from the centroblastic variant is not always straightforward and has generally met poor intraobserver and interobserver reproducibility. The anaplastic variant is rather

rare and is characterized by large cells with bizarre pleomorphic nuclei. These cells may resemble Hodgkin or RS cells and may show sinusoidal and/or cohesive growth pattern and even mimic undifferentiated carcinoma. The anaplastic morphology is independent of ALK expression in B-cell lymphomas. Other rare morphologic variants exist including the signet ring cell, microvillous and spindle cell variants.

Immunophenotype

The neoplastic cells characteristically express pan B-cell markers including CD19, CD20, CD22, PAX5 and CD79a. Surface and/or cytoplasmic immunoglobulin (IgM > IgG > IgA) can be demonstrated in 50–75% of the cases. Other markers commonly used in the characterization of DLBCL include CD10, BCL6, BCL2 and IRF4/MUM1 [1]. Aberrant phenotypes are not uncommon in DLBCL and may be responsible for confusion in the diagnosis. Lack of one or more B-cell markers may occur. In 10% of the cases the tumor cells express CD5. This CD5 positive DLBCL usually represent *de novo* cases, and only rarely are transformed CLL cases. Expression of CD5 is associated with worse prognosis even in the rituximab era. Aberrant expression of cytoplasmic CD3 has been also documented mainly in extranodal DLBCL without the expression of other T-cell markers. DLBCL with cyclin D1 expression lack the characteristic t(11;14) translocation of MCL, have a centroblastic morphology and a post-germinal center phenotype with positivity for IRF4/MUM1. These cases should be distinguished from MCL with blastic or pleomorphic morphology. *In situ* hybridization for EBVs should be performed in cases with geographic necrosis and/or Reed–Stenberg-like cells to exclude the possibility of an EBV+DLBCL of the elderly.

Determining the cell of origin

Because of the prognostic value of cell of origin and the increasing effort to tailor therapy based on molecular characteristics of DLBCL, a reliable method to identify GCB and non-GCB subtypes is needed. GEP, which is considered the gold standard to assign the molecular subtypes, is not routinely available and is not cost effective in routine diagnosis. Several studies have attempted to recapitulate the molecular subgroups (GCB vs. non-GCB) using a limited panel of antibodies available in most pathology laboratories (Figure 1). The Hans algorithm has been the most widely used in clinical trials. In this classifier three antibodies are used CD10, BCL6 and IRF4/MUM1. According to this algorithm DLBCL with CD10 expression in more than 30% cells belong to the GCB group. Cases that are CD10 negative, BCL6 positive but IRF4/MUM1 negative are also GCB subtype. Cases

that are IRF4/MUM1 positive with or without expression of BCL6 are assigned to the non-GCB subtype. There have been other algorithms attempting to improve the Hans classifier. These include the use of FOXP1 and GCET1 by Choi *et al.*, and the use of LMO2 by Natkunam *et al.* One study comparing different algorithms showed an 87% concordance with GEP for the Choi method and 86% for Hans scheme [7]. In this same study, the Tally algorithm based on FOXP1, GCET1, CD10, IRF4/MUM1 and LMO2 antibodies was demonstrated to be the most robust. The use of BCL6 was excluded from the analysis because it was considered a problematic, poorly reproducible stain. Nevertheless, in our experience BCL6 is reliable and helpful in the routine diagnosis. Accordingly, in the report from the international DLBCL rituximab-CHOP consortium program study, an algorithm based on expression of CD10, FOXP1 and BCL6 was used, which had a 92.6% concordance with GEP [8]. Although most studies find that immunohistochemical algorithms correlate with prognosis in DLBCL, everybody agrees that these algorithms are an imperfect substitution for GEP. The improvement of molecular techniques is making possible to use paraffin-embedded material with results comparable to fresh-frozen material. The recent development of a 20-gene assay using NanoString technology in paraffin-embedded tissue is a promising methodology with potential to be used as a routine method for determining cell of origin in DLBCL [9].

Prognostic importance of MYC and BCL2 in DLBCL

Translocations involving the *MYC* oncogene are the molecular hallmark of Burkitt lymphoma (BL). Most cases involve the *IGH@* gene on chromosome 14. Less commonly, the light chain genes on chromosome 2 and 22 are involved in the translocation. These translocations in BL are characteristically the sole chromosomal aberration identified [10,11]. Approximately 5 to 15% of DLBCL cases have been reported to carry *MYC* translocations, whereas 19–38% of cases show *MYC* low copy number gains [6]. Most *MYC* rearranged DLBCL cases fall into the GCB subtype [10,12]. In contrast to BL, *MYC* rearrangements in DLBCL are often seen to non-*IGH@* partners and with complex karyotypes [10]. The prognostic significance of *MYC* rearrangement alone in DLBCL is controversial. Several studies have demonstrated the association of *MYC* rearrangement with poorer outcome in DLBCL patients treated with R-CHOP [6]. However, recent studies have suggested that the impact of *MYC* is strongly influenced by *BCL2* and that *MYC* alone does not have a worse prognosis [13,14]. *MYC* rearranged DLBCL may either arise *de novo* or may represent a high-grade transformation of a low-grade lymphoma, usually follicular lymphoma. In the latter case the *MYC*

translocation is accompanied by a *BCL2* rearrangement, so-called ‘double-hit’ (DH) lymphoma. Approximately 40% of all DH lymphomas represent transformed FL cases [15]. However, 60–80% of *MYC* rearranged *bona-fide de novo* DLBCL are accompanied either by a *BCL2* and/or a *BCL6* rearrangement, representing DH or triple hit (TH) lymphomas [12,14]. Altogether DLBCL with DH/TH represent around 3–6% of all DLBCL and are generally refractory to standard chemotherapy regimens and have a poor prognosis [13,14,16]. Importantly, not all patients with DLBCL morphology and DH/TH may have a dismal prognosis. Recently, attention has focused on the apparent importance of the *MYC* partner because it has been suggested that only cases with an *IG/MYC* translocation have an adverse prognosis. The development of FISH assays readily performed in paraffin sections and more recently a monoclonal antibody that specifically recognizes MYC protein have facilitated the recognition of *MYC* alterations in routine diagnosis. Break-apart FISH probes are considered to be the most sensitive assay for identifying chromosomal rearrangements. In addition, the use of *MYC/IGH* dual fusion probe helps to confirm the *MYC* partner [6]. However, dual fusion probes for *MYC/IGκ* and *MYC/IGλ* are not commercially available.

MYC protein expression has been proposed as a rapid and cost-effective screening tool to identify *MYC* rearrangements. Although some studies agree that finding >70% MYC+ cells correlates with *MYC* rearrangement [6,13], a considerable number of cases with *MYC* rearrangement fall into the low *MYC* expression group (<40%) [12]. A combined approach *MYC*-FISH and IHC seems advisable. More common than *MYC* translocations is *MYC* protein overexpression reported to occur in 25–30% of DLBCL cases [13,14,16]. Most studies used a cut-off of 40% for *MYC* positivity. Of note, the negative prognostic impact of *MYC* protein expression is observed only in patients who simultaneously overexpressed *BCL2* protein, so-called ‘double-expresser’ (DE). The cut-off for *BCL2* protein overexpression varies in different studies, but most studies required at least 50% of tumor cells. *MYC* and *BCL2* DE have been reported to occur in 19–34% of DLBCL patients, and to have a worse prognosis than patients who do not express any or only one protein [13,14,16], but better prognosis than DH/TH DLBCL, which have a dismal outcome. Interestingly, the DE cases appear more common in the ABC subtype, and it has been suggested that this may largely contribute to the known inferior survival of the ABC subtype [16].

B cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL (BCLU)

The morphological distinction between BL and DLBCL has been problematic for pathologists. GEP studies have

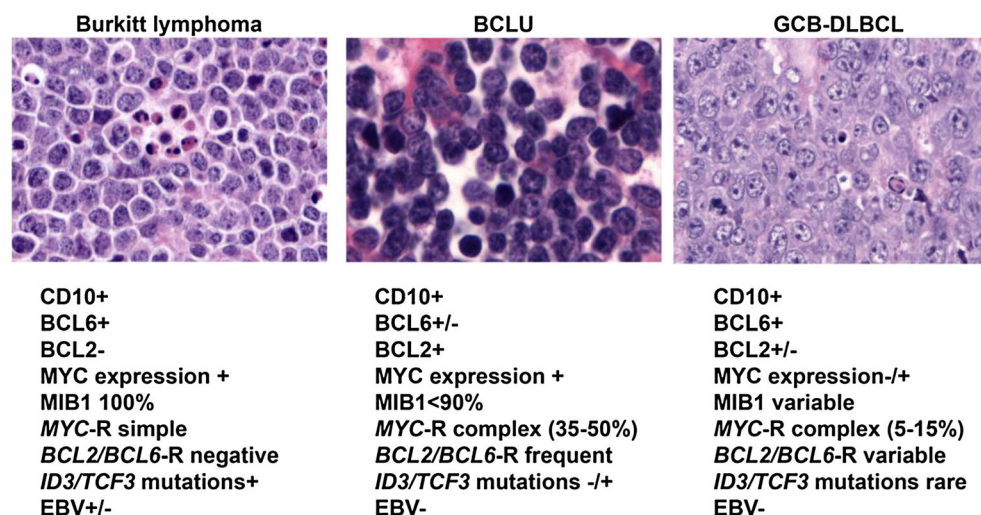


Figure 3. Differential diagnosis of Burkitt lymphoma, BCLU and DLBCL. Burkitt lymphoma is composed of medium-sized cells, with monotonous nuclei, inconspicuous nucleoli and moderate amount of cytoplasm. BCLU case composed of intermediate-size cells showing a rather monotonous nuclei, open chromatin and scant cytoplasm. DLBCL shows the characteristic cytological features of centroblasts (original magnifications 630×). The most frequent phenotype and genetic alterations in each group are shown

shown that BL has a characteristic signature but that there are cases within the spectrum of DLBCL and aggressive B-cell lymphomas, which have a similar molecular BL signature or fell into an intermediate category [10,11]. The 2008 WHO classification recognized this problematic and added a provisional category of B cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL (BCLU) (Figure 3) [15]. BCLU is a disease of older patients presenting with nodal or extranodal disease usually in an advanced clinical stage, high lactate dehydrogenase and frequent bone marrow and CNS infiltration with a dismal prognosis. Interestingly, *MYC* rearrangements can be detected in 30–50% of the cases usually associated with many more chromosomal aberrations [15]. The incidence of DH/TH in BCLU has been reported to be high (32%–78%). The precise morphological boundaries of this category are still not well defined. There is an ongoing discussion about how to classify aggressive lymphomas with DH/TH. One proposal is to continue classifying them based on morphology as DLBCL or BCLU or putting them together as a group of ‘DH/TH lymphomas’ regardless of the morphology of the tumor cells.

Conclusions

The understanding of the biology of DLBCL has increased tremendously in the last 15 years. The diagnosis of DLBCL needs to integrate, in addition to standard morphology and immunohistochemistry, all available ancillary techniques. The routine use of FISH and IHC to detect *MYC* and *BCL2* alterations/overexpression is recommended. Patients with DH and double expression of *MYC* and *BCL2* represent poor-risk subsets in which alternative strategies should

be explored [5]. The distinction of GCB versus ABC-DLBCL has not yet led to differences in primary treatment. The current standard of care for most patients is R-CHOP, which has improved dramatically the outcome of DLBCL. However, for patients who fail R-CHOP, the choice of therapy is very likely to be influenced by the cell of origin [5]. For example, bortezomib or BTK inhibitors have been shown to be effective in relapsed ABC-DLBCL but not in GCB subtype. On the contrary treatment with EZH2 inhibitors and BCL6 inhibitors have been shown to be effective in relapsed GCB-DLBCL. Emerging new targeted therapy will certainly influence the diagnosis and treatment of DLBCL in the near future.

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