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Zhao F. Xianfeng
Washington University School of Medicine in St. Louis

Anjum Hassan
Washington University School of Medicine in St. Louis

Arie Perry
Washington University School of Medicine in St. Louis

Yi Ning
University of Maryland - Baltimore

Sanford A. Strass
University of Maryland - Baltimore

See next page for additional authors

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Original Article

C-MYC Rearrangements are Frequent in Aggressive Mature B-Cell Lymphoma with Atypical Morphology

Xianfeng F. Zhao[1, 2], Anjum Hassan[1], Arie Perry[1], Yi Ning[2], Sanford A. Stass[2] and Louis P. Dehner[1]

[1]*Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA and*
[2]*Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, USA*

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Abstract: Diagnosis and classification of aggressive mature B-cell lymphoma with atypical morphology remains a challenge. To identify factors that may contribute to the atypical morphology, we selected eight such cases and evaluated their morphologic, immunophenotypic and cytogenetic features and clinical outcomes. The neoplastic cells showed a diffuse monotonous infiltrating pattern with a spectrum of morphology including: 1) L1 lymphoblastic; 2) centroblastic; 3) immunoblastic; and 4) mixed centroblastic and immunoblastic. The lymphoma cells in most cases were positive for CD10 and/or BCL6, and showed BCL2 expression. 6 of 8 cases showed C-*MYC* rearrangements, and interestingly, all 6 cases demonstrated a proliferation index of $\leq$90%. 3 of the 6 cases also demonstrated t(14;18). Clinical follow-up indicated that aggressive mature B-cell lymphoma may benefit from more intensified chemotherapeutic regimens used for BL. Our study suggests that aggressive mature B-cell lymphoma with atypical morphology may be another "grey zone lymphoma" lying in the spectrum between Burkitt lymphoma and diffuse large B-cell lymphoma.
Key Words: Aggressive mature B-cell lymphoma, Burkitt lymphoma, diffuse large B-cell lymphoma, grey zone lymphoma, *C-MYC* rearrangement

Introduction

Classical Burkitt lymphoma (BL) is characterized morphologically by a diffuse proliferation of monotonous medium-sized lymphoma cells with round nuclei, small nucleoli and high proliferative as well as apoptotic indices. Interspersed in the sea of round "blue" cell tumor are Tingible-body macrophages with abundant clear cytoplasm and apoptotic bodies, imparting a "starry-sky" pattern of the neoplasm at low magnification [1]. BL is invariably associated with C-MYC over-expression as a result of *C-MYC* gene rearrangements. In contrast, diffuse large B-cell lymphoma (DLBCL) includes a heterogeneous group of intermediate to high-grade mature B cell neoplasms. The lymphoma cells are usually larger and show more variation in cell size and the amount of cytoplasm with vesicular chromatin and more prominent nucleoli [2].

Because of the drastic difference in molecular mechanism, treatment regimens and clinical outcomes, it is imperative to correctly diagnose and classify BL or DBLCL. With the help of immunophenotyping and cytogenetics, it is usually possible to differentiate classical BL from typical DLBCL. Both lymphomas are positive for all the mature B-cell markers, and sometimes CD10 and BCL6. Classical BL has a nearly 100% proliferation index and is always negative for BCL2 [3]. On the other hand, DLBCL in general shows a <90% proliferation index and is frequently positive for BCL2 [4]. In addition, *C-MYC* gene rearrangements are present in virtually all classical BLs, but only in a minority of DLBCLs.

Problem arises when morphology of the lymphoma cells is not characteristic of either BL or DLBCL, herein referred to as aggressive mature B-cell lymphoma with atypical morphology. On one hand, the lymphoma cells are monotonous resembling classical BL or lymphoblastic lymphoma; however, they usually have more abundant cytoplasm, irregular nuclei, and sometimes more prominent nucleoli, consistent with the

cytological features of DLBCL. On the other hand, these cells do not show the variations in size and shape usually seen in typical DLBCL. Recognizing this problem, a provisional entity of "atypical Burkitt/Burkitt-like lymphoma" was introduced to define some of these cases with: 1) a characteristic BL immunophenotype; 2) a nearly 100% proliferation index; and 3) consistent presence of *C-MYC* translocations in the most recent WHO lymphoma classification [2]. Cases that do not fulfill all three criteria are currently considered to be DLBCL. However, more recent molecular evidence indicates that these criteria do not completely differentiate BL from DLBCL, and a reproducible distinction between BL and DLBCL is not always possible [5].

In this small series, we present 8 cases of aggressive mature B-cell lymphoma with atypical morphology. Although these cases may fall into the category of DLBCL by the current WHO classification, due to their atypical features and high clinical suspicion for BL, these cases were extensively analyzed by immunohistochemistry and fluorescence in situ hybridization (FISH). The results indicate that these cases may represent another "grey zone lymphoma" lying in the spectrum between BL and DLBCL.

Materials and Methods

Selection of Cases

All cases in this study were selected from the archives of the Department of Pathology, Barnes-Jewish Hospital and the Laboratories of Pathology, University of Maryland Medical Center from July 2004 to June 2006. This selection was based on the clinical suspicion of BL, atypical morphology and available cytogenetic data. None of the patients had a documented history of HIV infection. The Institutional Review Boards from both institutions have approved the study.

Tissue Sources

Cervical lymph node or bone marrow biopsies were performed on most patients, with one half of the specimens fixed in 10% formaldehyde and the other half submitted for immunophenotypic analysis. Tissue sections were prepared from the formalin-fixed and paraffin-embedded biopsies using standard techniques. The sections were stained with routine hematoxylin-eosin (H&E). The morphology was reviewed independently by two hematopathologists. When there was discrepancy, FISH studies using the *C-MYC* break-apart and *IGH/BCL2* dual fusion probe sets were performed. A consensus diagnosis was reached based on morphology, immunophenotype and the status of *C-MYC* rearrangement in the tumor with clinical correlation.

Immunohistochemistry

Immunohistochemical stains were performed using a Biotek Techmate 1000 autostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's protocol. Mouse anti-human CD3, CD5, CD20, CD79a, BCL2, BCL6, Ki-67, MUM-1 and TdT (DAKO Corporation, Carpenteria, CA) were used as the primary antibodies. Horseradish peroxidase-labeled rabbit anti-mouse polyclonal antibodies were employed to convert the chromogen 3, 3'-diaminobenzidine tetrahydrochloride substrate. All stains were performed with appropriate positive and negative controls. The proliferation indices were expressed as percentages and calculated using the formula (number of Ki-67+ cells/number of CD20+ cells) x 100. The number of CD79a+ cells was used in the formula for the CD20-negative case (patient 3). Background T-cells were excluded from assessment of the proliferation indices.

Flow Cytometric Immunophenotyping

Immunophenotyping was performed using the FC500 four-color flow cytometer (Beckman-Coulter, Miami, FL) according to the standard protocol (Becton-Dickinson, San Jose, CA). Directly conjugated monoclonal antibodies (Beckman-Coulter, Miami, FL) to the following antigens were used for this analysis: CD3 (UCHT-1), CD4 (SFC112T4D11), CD5 (SFC124T6G12), CD7 (8H8.1), CD8 (SFC121Thy2D3), CD10 (J5), CD19 (PC5), CD20 (B9E9), CD23 (HD50), CD45 (J.33), CD79a (HM47), FMC7 (FMC7), HLA-DR (Immu-357) and terminal deoxynucleotidyl transferase (TdT) (HT1+HT4+HT8+HT9). Directly conjugated monoclonal antibodies against CD2 (55.2) were obtained from Becton-Dickinson, San Jose, CA. Polyclonal rabbit anti-human immunoglobulin light chains were obtained from DakoCytomation, Carpenteria, CA. All antibodies were

conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PCP) or allophycocyanin (APC). The flow cytometric data was analyzed using CellQuest software (Becton-Dickinson, San Jose, CA).

Fluorescence In Situ Hybridization (FISH) Analysis

Dual-color FISH was performed either on lymph nodes or on cells from bone marrow as previously described [6]. Commercially available dual color break-apart *C-MYC* and dual fusion *IGH/BCL2* probe sets were used following the manufacturer's instructions (www.Vysis.com). The LSI MYC Dual Color, Break Apart Rearrangement Probe is a mixture of two probes that hybridize to opposite sides of the *C-MYC* gene (The SpectrumOrange™ labeled 5' LSI MYC probe begins upstream of the 5' end of *C-MYC* and extends 260 kb toward the centromere. The SpectrumGreen™ 3' LSI MYC probe starts about 1 Mb 3' of *C-MYC* and extends toward the telomere for about 400 kb) (www.Vysis.com). This probe set can detect the *C-MYC* rearrangements involved in almost all the breakpoints for t(8;14)(q24;q32), t(8;22)(q24;q11) and t(2;8)(p11;q24). For FISH on lymph nodes, thin sections (5-6 μm) from formalin-fixed paraffin-embedded block were mounted on poly-L-lysine–coated slides. After deparaffinization, the sections were subjected to "antigen retrieval" using "steam cooking" in citrate buffer for 20 minutes, followed by re-hydration. After pepsin digestion at 37°C for 30 minutes and a subsequent wash in 2 x SSC, the slides were allowed to air dry and followed by FISH analysis. After the hybridization, nuclei were counterstained with 4′6-diamidino-2-phenyl-indole·2HCl (0.5 µL/mL), and the sections were viewed under an Olympus BX60 fluorescent microscope with appropriate filters (Olympus, Melville, NY). Hybridizations were digitally photo-graphed using a high-resolution COHU CCD black-and white camera, with a Z-stack motor programmed to capture images at sequential 4′6-diamidino-2-phenylindole·2HCl (1 level), fluorescein isothiocyanate (5 levels), and rhodamine (5 levels) filter settings. Reconstruction into a single superimposed image with blue, green, and red pseudocolors was accomplished using the CytoVision workstation software (Applied Imaging, Santa Clara, CA).

Results

There were four in-house (patients 1, 3, 4 and 7) cases and four outside consultation (patients 2, 5, 6 and 8) cases in this study. There was equal number of male and female patients. Their age ranged from 19 to 71 years with a median age of 63. The patient information, treatment regimens and clinical follow-up are summarized in **Table 1**.

All lymphomas were composed of a monotonous population of neoplastic cells resembling BL. However, they demonstrated various atypical morphologic features often seen in DLBCL. These included L1 lymphoblastic, centroblastic, immunoblastic, and mixed centroblastic and immunoblastic morphology (**Figure 1**). Unlike typical DLBCL, these cases showed less variation in size and shape of the neoplastic cells. Some of the neoplastic cells have abundant basophilic cytoplasm, cytoplasmic vacuoles and prominent nucleoli (**Figure 2**).

As summarized in **Table 2**, the lymphoma cells expressed CD20 in all but one cases. The lymphoma cells in the one CD20-negative case were positive for CD79a and expressed surface immunoglobulin light chain. BCL2 was positive in 5 of 6 cases that had *C-MYC* rearrangements. The lymphoma cells were either positive for both CD10 and BCL6 (3/8), or at least one of these two markers (4/8), indicating their germinal center origin. Interestingly, however, MUM-1 was positive in all 4 cases analyzed and in 2 of the 4 cases the lymphoma cells were positive for CD10. The lymphoma cells in 6 of 8 cases showed a $\leq$95% proliferation index. Extensive immunohisto-chemical studies were not performed on case 5 due to the lack of additional tissue.

With the break-apart *C-MYC* probe, we identified the presence of *C-MYC* rearrangements in all 6 cases with $\leq$90% proliferation indices (**Figure 3** and **Table 2**). Conversely, no *C-MYC* rearrangement was detected in the remaining 2 cases with $\geq$95% proliferation rates. Although *C-MYC* rearrangement is characteristic of BL, it has also been identified in rare cases of DLBCL [4, 7]. In contrast, t(14;18) was present in about 30% of DLBCL, but was never detected in classical BL [8]. To further characterize these

Table 1 Clinical information of the patients

Case #	Age/Sex	Diagnosis	Treatment	Clinical outcome
1	52/M	Large B-cell lymphoma, stage IA; Status post failed autotransplant; Recurrence in CNS	High dose methotrexate, ARA-C, Rituxan; intrathecal methotrexate and ARA-C	Alive (8 years) with refractory disease; in hospice
2	67/F	High-grade large B-cell lymphoma	Combined chemotherapy and radiation therapy	Alive (22 months) with no evidence of disease
3	71/M	Low-grade B-cell lymphoma for 8 years; High-grade B-cell lymphoma	Salvage chemotherapy with CHOP	Died of refractory disease (8 months)
4	64/F	Large B-cell lymphoma	CHOP+Rituxan, subsequently changed to CALGB trial with intrathecal therapy	Alive (12 months) with no evidence of disease
5	19/M	Diffuse large B-cell lymphoma, stage IV	Reduction with vincristine, prednisone and cyclophosphamide for 7 days; Induction with vincristine, prednisone, methotrexate, cyclophosphamide and doxorubicin for 7 days; Consolidation with methotrexate, hydrocortisone, ARA-C and prednisone	Alive (24 months) with no evidence of disease
6	71/M	High-grade B-cell lymphoma	CHOP+Rituxan x 4 cycles	Alive (3 months)
7	62/F	Atypical Burkitt lymphoma	High dose chemotherapy involving methotrexate with leukovorin rescue	Died primarily of renal insufficiency 4 months after diagnosis
8	36/F	Diffuse large B-cell lymphoma	CHOP+Rituxan x 6 cycles	Alive (18 months) with no evidence of disease

Table 2 Characteristics of aggressive mature B-cell lymphomas with atypical morphology

Case #	Immunophenotype							Cytogenetics/FISH results		
	CD20	CD79a	CD10	BCL2	BCL6	MUM-1	KI-67	C-MYC	IHG/BCL2	Others
1	+	NT[a]	+	+	+	+	~50%	+	+	Polysomy 8
2	+	+	-	+	+	NT	~90%	+	NT	None
3	-	+[b]	+	+	-	+	~70%	+	+	None
4	+	-	-	+	+	+	~90%	+	-	Polysomy 8
5	+	NT	NT	NT	NT	NT	~50%	+	-	None
6	+	NT	+	+	+	NT	~90%	+	+	None
7	+	+	+	+	+	NT	~100%	-	NT	None
8	+	NT	-	-	+	+	~95%	-	-	None

[a]NT: Not tested; [b]by flow cytometry

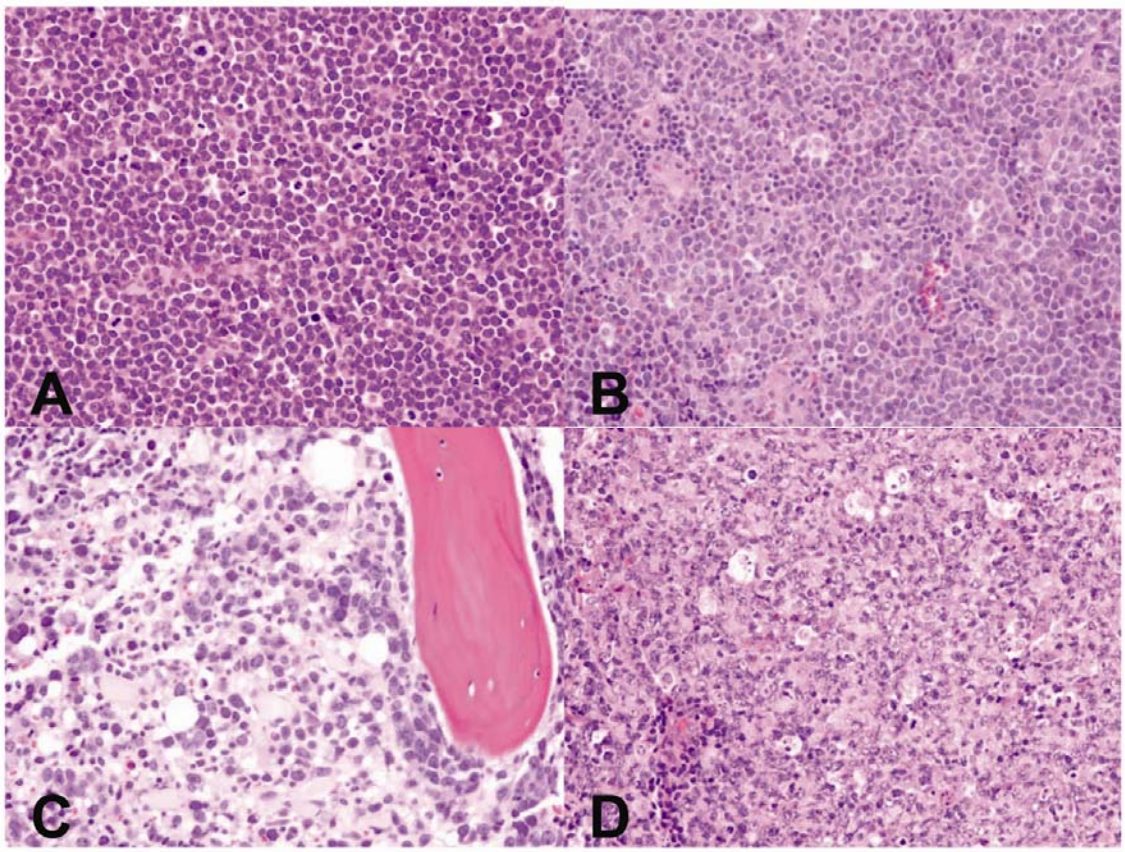

Figure 1 Morphologic variations in aggressive mature B-cell lymphomas with atypical morphology (H&E, x 400 magnification). **A.** L1 lymphoblastic morphology: a monotonous population of medium-sized neoplastic cells with scant cytoplasm, round nuclei and small nucleoli (from patient 3); **B**. Centroblastic morphology: large cells with abundant cytoplasm, vesicular nuclei and small nucleoli; **C**. Immunoblastic: large cells with abundant cytoplasm and single centrally located prominent nucleolus; **D**. Centroblastic and immunoblastic morphology: with features of (**B**) and (**C**).

lymphomas, FISH studies were performed on all cases to evaluate the presence of *IGH/BCL2* fusion gene associated with t(14;18). The results showed that *IGH/BCL2* was present in 3 of the 6 cases with *C-MYC* rearrangements and ≤90% proliferation indices (**Table 2**). However, t(14;18) was not detected in the remaining 2 cases with ≥95% proliferation indices and absent *C-MYC* rearrangements. As controls, FISH was also performed in two additional cases of classical BL and two additional cases of typical DLBCL. None of these cases harbored the t(14;18)(data not shown).

The therapeutic regimens were modified accordingly based on the FISH results. Two cases (cases 1 and 4) with *C-MYC* rearrangements responded poorly to Rituximab (R), Cyclophosphamide (C), Adriamycin (H), Vincristine (O) and Prednisone (P) (R-CHOP) previously, but later responded favorably to intensified chemotherapy (**Table 1**). After *C-MYC* rearrangement was identified, two cases (cases 2 and 5) were treated with either combined chemotherapy/radiation therapy or intensified chemotherapy; both are currently in remission for almost two or over two years. One patient (case 3) with *C-MYC* rearrangement failed salvage CHOP therapy and died, and another (case 6) was followed for only 3 months after R-CHOP. *C-MYC* rearrangements were not detected in two patients: one responded well to R-CHOP and remained disease-free for 18 months (case 8); the other was treated with high dose chemotherapy including methotrexate and died of renal failure 4 months after the diagnosis (case 7). Although the number of patients is small, it appears that patients with

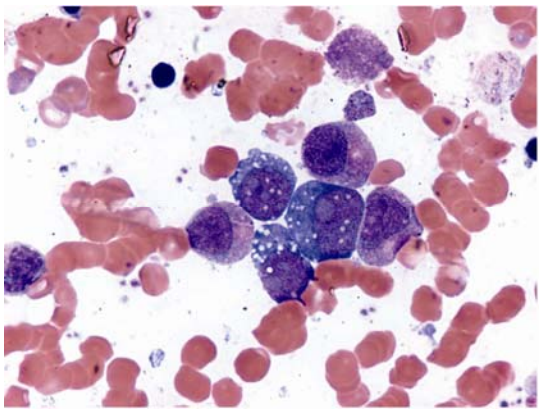

Figure 2 Aggressive mature B-cell lymphoma with atypical morphology in bone marrow (from patient 6). The bone marrow aspirate showed several large lymphoid cells with abundant basophilic and vacuolated cytoplasm, irregular nuclei and multiple prominent nucleoli (Wright-Giemsa, x 1000 magnification). These cells have sizes similar to the adjacent promyelocyte and myelocytes.

aggressive mature B-cell lymphoma with atypical morphology and *C-MYC* rearrangements have benefited from the intensified chemotherapeutic regimens for BL rather than R-CHOP for DLBCL.

Discussion

We retrospectively evaluated 8 cases of aggressive mature B-cell lymphoma with atypical morphology during a two-year period from two medical centers. *C-MYC* rearrangements are frequently found in these lymphomas (6/8, 75%). In addition, these lymphomas also frequently harbor t(14;18) (3/5, 60%) and/or trisomy 8 (2/6, 20%) genetic abnormalities. The current study indicates that these genetic abnormalities might be associated with the atypical morphology of aggressive B-cell lymphomas. Interestingly, *C-MYC* rearrangements are identified in all 6 cases with $\leq$90% proliferation index, but are not detected in the remaining 2 cases with $\geq$95% proliferation index, demonstrating a poor correlation between the proliferation index and *C-MYC* rearrangements. Although this series is small, the results demonstrate the potential pitfall of using a near 100% proliferation rate as a surrogate marker for the diagnosis of BL. With overlapping features of BL and DLBCL, these aggressive mature B-cell lymphomas with atypical morphology may be another "grey zone lymphoma" lying between BL and DLBCL, and may require molecular studies to further define them [5, 9, 10].

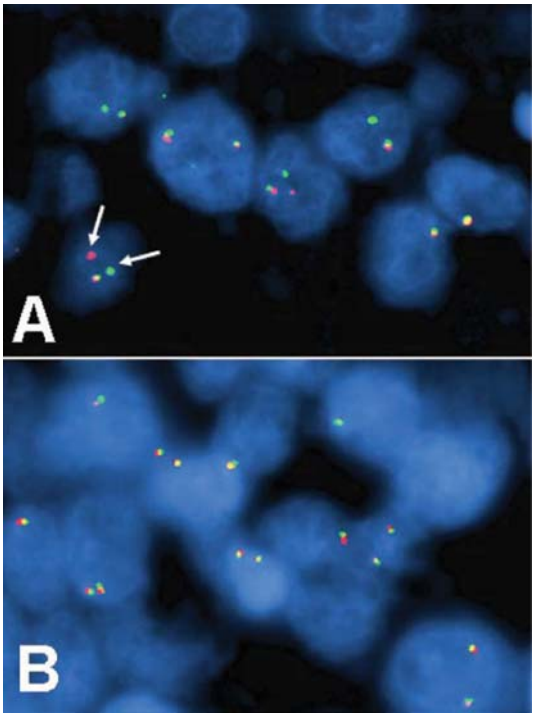

Figure 3 FISH analysis using the c-*MYC* break-apart probe set. **A**. A c-*MYC* rearrangement is present (patient 1) as indicated by splitting apart of the normal red-green fusion signal into one red (centromeric end of *MYC*) and green (telomeric end of *MYC*) signal (arrows); **B**. An c-*MYC* rearrangement is absent (patient 8) as evidenced by the presence of normal red-green fusion signals only.

How should these cases be classified based on the current information available? In addition to the neoplastic cells having a mature B-cell phenotype, they also express BCL2 as well as CD10 and/or BCL6, which are markers for germinal center B cells. Both BL and some of the DLBCL are believed to originate from the germinal center B-cells based on their expression of CD10 and/or BCL6 [11]. It is also believed that BCL2 expression can only be detected in DLBCL, but not in BL [2-4]. However, Barth *et al* recently compared 7 endemic and 7 sporadic BL cases [12]. They found that a uniform expression of CD10 was seen only in endemic BL cases (7/7), and about half of the sporadic BL cases (4/7) were negative for CD10. One of the sporadic BL case also expressed BCL2 (1/7). A recent study of 220 aggressive mature B-cell

lymphomas demonstrated that >20% of the molecular Burkitt lymphomas expressed BCL2 [5]. In our study, most cases were positive for both BCL2 (6/7) and *C-MYC* rearrangements (6/8). Although the majority of our cases are BCL6-positive (6/7), almost half of our cases (3/7) are negative for CD10 (see **Table 2**). Considering the clinical behavior, the cases with *C-MYC* rearrangements (6/8) might have been atypical sporadic BL in retrospect.

The t(8;14) and its variant chromosomal translocations are currently the most specific cytogenetic abnormalities for BL [3]. FISH analysis is one of the most sensitive approaches in identifying all these translocations in routine formalin-fixed paraffin-embedded tissue sections. However, the commonly used *MYC/IGH* probe can only detect the fusion in ~80% of the BL [13, 14]. Recently, the break-apart *C-MYC* probe set (Vysis) was developed to detect t(8;14) as well as the variant *C-MYC* rearrangements t(2;8) and t(8;22)[15]. We opted to use this probe set in this study because of its increased diagnostic sensitivity. Although the *C-MYC* partner genes are not identified by this approach, since *IG-MYC* is much more common in aggressive mature B-cell lymphomas [9], most of the partner genes in our cases are presumably *IG*. Since *C-MYC* gene may be activated by translocations with other partner genes [16], our approach may be able to detect those *C-MYC* rearrangements as well. With our approach, we have detected *C-MYC* rearrange-ments in most (75%) of our cases of morphologically aggressive mature B-cell lymphomas. The findings are clinically relevant because more and more studies suggested that DLBCL with *C-MYC* rearrangements might have a clinical course resembling BL [17, 18].

Half of our cases harbor the t(14;18) in addition to *C-MYC* rearrangements, which created a dilemma in classifying these lymphomas. Although lymphomas similar to our cases were lumped together with DLBCLs and the *C-MYC* rearrangement was considered a secondary event reflecting tumor progression [19], recent studies suggested that DLBCLs with both *C-MYC* rearrangements and t(14;18) behaved more aggressively clinically like BL [17, 18]. Therefore, even if the *C-MYC* rearrangement is indeed an event of progression, the emergence of t(8;14) in addition to the t(14;18) should perhaps justify the interpretation of progression to BL [20], and thus the lymphoma should be managed like BL. Further studies are needed to characterize mature B cell lymphomas with both *C-MYC* rearrangement and t(14;18) since their clinical management may be significantly different from either BL or DLBCL.

To avoid under- or over-treatment of the patients, it is critical to distinguish BL from DLBCL when encountering these aggressive mature B-cell lymphomas with atypical morphology. Since diagnosis of BL requires strict criteria (CD10+, BCL6+, close to a 100% proliferation index and *IG-MYC*), many cases that do not meet all these criteria were diagnosed as DLBCL, and therefore would not receive the benefit of intensified chemotherapy. More recent studies [5, 9, 10] suggest that some of those DLBCL cases indeed had the molecular signatures of BL and over 10% of those molecular BL did not harbor any detectable *C-MYC* translocations [4].

In summary, we should recognize this group of aggressive mature B-cell lymphomas with atypical morphology and *C-MYC* rearrange-ments when determining chemotherapy, since more and more studies show that they follow a similar clinical course as BL [17, 18]. Although molecular profiling may be the eventual solution [9, 10], it is not yet ready for real-time diagnosis [21]. Cytogenetic studies may be routinely performed in all aggressive mature B-cell lymphomas with atypical morphology. With the increasing number of reported cases [5, 9, 10, 17, 18], "aggressive mature B-cell lymphomas with atypical morphology" may emerge as another "grey zone lymphoma" between BL and DLBCL, and multicenter collaborative investigation is essential to further define the clinical, pathological and molecular signatures of these lymphomas.

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Please address all correspondences to Dr. XianFeng F. Zhao, MD, PhD, Hematopathology

Section, Department of Pathology, University of Maryland School of Medicine, 22 S Greene Street, NBW78, Baltimore, MD 21201, USA. Tel: 410-328-5555; Fax:410-328-5508; Email: xzhao@umm.edu.

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