# Germinal Center and Activated B-Cell Profiles Separate Burkitt Lymphoma and Diffuse Large B-Cell Lymphoma in AIDS and Non-AIDS Cases

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## Abstract

*Morphologic features of Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL) overlap. No single phenotypic marker or molecular abnormality is pathognomonic. We tested a panel of 8 germinal center (GC) and activated B-cell (ABC) markers for their ability to separate BL and DLBCL. We diagnosed 16 BL and 39 DLBCL cases from 21 patients with AIDS and 34 without AIDS based on traditional morphologic criteria, Ki-67 proliferative index, and c-myc rearrangement (fluorescence in situ hybridization). After immunohistochemically staining tissue microarrays of BL and DLBCL for markers of GC (bcl-6, CD10, cyclin H) and ABC (MUM1, CD138, PAK1, CD44, bcl-2), we scored each case for the percentage of positive cells. Hierarchical clustering yielded 2 major clusters significantly associated with morphologic diagnosis (P < .001). For comparison, we plotted the sum of the GC scores and ABC scores for each case as x and y data points. This revealed a high-GC/low-ABC group and a low-GC/high-ABC group that were associated significantly with morphologic diagnosis (P < .001). Protein expression of multiple GC and ABC markers can separate BL and DLBCL.*

Because the clinical behavior and treatment differ significantly between Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL),[1-5] accurate diagnosis is important. Histologic subclassification can be difficult because morphologic features overlap.[6] Furthermore, no single immunologic marker or molecular abnormality definitively separates BL from DLBCL. For example, both lymphoma types can express B-cell markers, CD19, CD20, and CD22,[6] and germinal center (GC) markers, CD10 and bcl-6 protein, and can translocate the c-*myc* oncogene.[7-10]

Microarray studies of complementary DNA, oligonucleotides, and protein expression divide the DLBCL morphologic category into a GC type, an activated B-cell (ABC) type, and a third type with neither a GC nor an ABC pattern, called type 3. Among DLBCLs, the GC type predicts a superior survival compared with the ABC type or type 3.[3-5,11-13] Gene microarrays also can divide DLBCLs into other clinically relevant subclassifications.[14]

Numerous investigators have suggested that BL arises from a germinal center B cell,[6] but, to our knowledge, the GC-ABC protein expression profiles of BL and DLBCL have not been compared directly. We tested a panel of 8 GC and ABC immunohistochemical markers to determine whether they could separate BL from DLBCL. In addition, we compared the sensitivity and specificity of conventional hierarchical clustering against a new analytic method using a 2-dimensional (2D) contour-frequency plot.

## Materials and Methods

### Lymphoma Samples

We retrospectively studied 56 DLBCLs: 23 AIDS-related and 33 non–AIDS-related cases; and 16 BLs: 9 AIDS-related

and 7 non–AIDS-related cases. These cases were retrieved from the archives of Montefiore Medical Center, Bronx, NY; Lenox Hill Hospital, New York, NY; and Hackensack Medical Center, Hackensack, NJ. The cases were selected on the basis of morphologic diagnosis, availability of paraffin blocks, and clinical follow-up. Permission to use these samples for research was granted by the respective institutional review boards. We excluded 17 cases of DLBCL because of previous therapy or a preceding low-grade lymphoma. The remaining de novo non-Hodgkin lymphomas consisted of 39 DLBCL and 16 BL cases, which were classified morphologically according to the World Health Organization (WHO) criteria.[6] BL was further subdivided as classic or variant types and DLBCL as centroblastic or immunoblastic types.

## Clinical Information

The following clinical data were obtained: sex; International Prognostic Index score, which includes the following factors: age, serum lactate dehydrogenase, nodal vs extra nodal presentation, performance status, and stage; AIDS status; and progression-free and overall survival. The study was performed according to the institutional review board guidelines of the participating institutions.

## Tissue Microarrays

Tissue microarrays (TMAs) were constructed from formalin-fixed, paraffin-embedded tissue blocks using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD). Each case was represented in triplicate using 1.0-mm cores.[15] Reactive tonsil, liver, and kidney tissues were included as control samples.

## Immunohistochemical Analysis

We stained the lymphoma samples embedded in the TMAs with a panel of GC (bcl-6, CD10, and cyclin H) and ABC (MUM1, CD138, PAK1 [p21 activated kinase 1], CD44, and bcl-2) markers. We chose cyclin H and PAK1 because they are different from other traditional GC and ABC markers previously studied and they represent 2 regulatory pathways. PAK1 has been shown to have roles in gene transcription, cell morphology, motility, and apoptosis.[16] Cyclin H has roles in DNA repair and cell cycle regulation.[17] In addition, we assessed T-cell (CD3), B-cell (CD20), and proliferation (Ki-67) markers. Immunohistochemical analysis was performed using an avidin-biotin technique with modifications as noted in ▌Table 1▐.

Briefly, 4- to 6-μm-thick sections of formalin-fixed, paraffin-embedded tissue blocks were deparaffinized, rehydrated, blocked with 3% hydrogen peroxide, appropriately retrieved, and incubated with the primary antibody, followed by use of the secondary detection system using diaminobenzidine with the LSAB2 system (Serotec, Oxford, England) or the EnVision system (DakoCytomation, Carpinteria, CA). We performed the CD3, CD20, and CD138 immunostains using a DakoCytomation Autostainer; the other antibodies were stained manually. Tonsil was used as a positive control sample for CD3, CD10, CD20, CD138, MUM1, bcl-2, bcl-6, and Ki-67; additional positive control tissue samples were adult human testes for cyclin H and invasive mammary ductal carcinoma for PAK1.

We scored each immunostain for the percentage of positive cells in a semiquantitative manner as follows: 0, 0% to 19%; 1, 20% to 49%; and 2, 50% to 100%. We did not evaluate stain

▌Table 1▐
**Antibodies Used for Tissue Immunohistochemical Analysis**

| Antibody | Source | Clone | Dilution | Specificity | Retrieval | Detection System |
|---|---|---|---|---|---|---|
| CD3[*] | Mouse monoclonal | F2.2.38 | 1:20 | — | ER[†] | EnVision Monoclonal[‡] |
| CD10[§] | Mouse monoclonal | — | 1:20 | — | HipH[∥] | EnVision Monoclonal[‡] |
| CD20[¶] | Mouse monoclonal | L26 | 1:50 | — | ER[†] | EnVision Monoclonal[‡] |
| CD44[#] | Mouse monoclonal | DF1485 | 1:40 | — | TR[**] | EnVision Monoclonal[‡] |
| CD138[#] | Mouse monoclonal | B-B4 | 1:500 | — | ER[†] | EnVision Monoclonal[‡] |
| BCL-2[#] | Mouse monoclonal | 124 | 1:100 | — | HipH[∥] | EnVision Monoclonal[‡] |
| BCL-6[#] | Mouse monoclonal | PG-B6p | 1:20 | Cow, rabbit, rat, sheep, swine | HipH[∥] | EnVision Monoclonal[‡] |
| Cyclin H[††] | Mouse monoclonal | D-10 | 1:100 | Human, mouse, rat | ER[†] | LSAB2 system[#] |
| Ki-67[‡] | Mouse monoclonal | MIB-1 | 1:50 | Human, cow, dog, horse, sheep, swine | TR[**] | EnVision Monoclonal[‡] |
| MUM1 (IRF4)[††] | Goat polyclonal | M-17 | 1:200 | Human, mouse, rat | TR[**] | Goat ABC staining system[††] |
| PAK1[‡‡] | Rabbit polyclonal | — | 1:50 | Human, mouse, rat, monkey, guinea pig | ER[†] | EnVision Polyclonal[‡] |

ABC, avidin-biotin-peroxidase complex.
[*] BioGenex, San Ramon, CA.
[†] Epitope Retrieval Solution, citrate pH 6, DakoCytomation, Carpinteria, CA.
[‡] DakoCytomation, Carpinteria, CA.
[§] Cell Marque, Hot Springs, AR.
[∥] High pH Target Retrieval Solution, pH 9.9, DakoCytomation, Carpinteria, CA.
[¶] Zymed, San Francisco, CA.
[#] Serotec, Oxford, England.
[**] Target Retrieval Solution, pH 9, DakoCytomation, Carpinteria, CA.
[††] Santa Cruz Biotechnology, Santa Cruz, CA.
[‡‡] Cell Signal, Beverly, MA.

intensity. For MUM1, bcl-6, and Ki-67, we considered only nuclear staining. We stratified the percentage of Ki-67–positive cells as follows: 0, 0% to 49%; 1, 50% to 79%; and 2, 80% to 100%. The Ki-67 percentage represents all cells. We chose 80% as a lower cutoff for the Ki-67 upper range because we obtained paraffin blocks from multiple institutions, some more than 10 years old, with different lengths of fixation and variable storage conditions.

### Fluorescence In Situ Hybridization for c-*myc* Translocation

Translocations involving the c-*myc* locus were detected with an LSI MYC Dual Color, Break Apart Rearrangement Probe (Vysis, Downers Grove, IL), comprising 2 probes that flank opposite sides of the region 3' of *myc*. One probe begins upstream of the 5' end of the *myc* locus (extending 260 kilobases toward the centromere), and the other starts 1 megabase 3' of the *myc* locus (extending 400 kilobases toward the telomere).

We performed fluorescence in situ hybridization (FISH) on formalin-fixed, paraffin-embedded tissue sections according to the manufacturer's directions with the following modifications: deparaffinization, Hemo-De clearing agent (Fisher Scientific, Pittsburgh, PA), 15 minutes; pretreatment, sodium thiocyanate, 20 minutes at 80°C; protease digestion, 14 minutes at 37°C; sample fixation (to the glass slide), 10% buffered formalin, 10 minutes at room temperature; dehydration in increasing concentrations of ethyl alcohol, 70%, 85%, and 100%, for 1 minute each at room temperature; probe preparation; slide preparation and denaturization, 6 minutes at 80°C; hybridization; and interpretation. The slides were evaluated using chroma spectrum orange and spectrum green filters on a Zeiss Axioskop (1,000×) (Carl Zeiss, Oberkochen, Germany). The images were captured with a COHU monochrome CCD camera and analyzed with Macprobe software (Applied Imaging/PSI, Santa Clara, CA). A normal nucleus hybridizing with the probe displayed 2 yellow (orange/green) fusion signals, whereas a nucleus with a c-*myc* translocation involving t(2;8), t(8;22), or t(8;14) displayed 1 orange, 1 green, and 1 yellow (fusion) signal. To optimize the assay, we used known samples of BL. To standardize the assay, we used a TMA composed of triplicate cores from 30 reactive tonsils.

### Statistical Methods

#### Hierarchical Cluster Analysis

We used "Cluster," a hierarchical clustering program,[18] to analyze the expression of 8 GC and ABC immunohistochemical markers among 16 BL and 39 DLBCL cases. Although the software originally was designed for clustering complementary DNA microarray data, it recently was applied to a group of tumors based on immunohistochemical TMAs.[19,20] We used hierarchical clustering to order data in 2 dimensions.

First, the clustering software groups tumors based on the relatedness of the immunohistochemical staining using the 8 antibodies; second, it groups the antibodies according to their relatedness across all cases. After we generated the clustered data, we graphically displayed the information as heat maps and dendrograms using the "Treeview" program. The Cluster and Treeview programs are freely available on the Internet (http://rana.lbl.gov/EisenSoftware.htm). We prepared the microarray input data files and ran the Cluster program according to the method of Liu et al.[20]

#### Contour-Frequency Plots

The individual scores for the ABC markers and GC markers were summed for each case, yielding total ABC and total GC scores. Then, a 2D grid was constructed with the ABC totals plotted along the x-axis and the GC totals plotted along the y-axis. By assigning a coordinate pair (ABC, GC) to each case, the overall protein expression profile could be assessed as a single data point. To help visualize any clustering in the set of coordinate data points, we constructed a 2D contour-frequency plot; the number of cases is proportional to the gray intensity.

#### Other Statistical Methods

We compared the distributions of data groups (clinical data and cluster data) by using the Fisher exact test. We compared means of ages between clinical groups using the unpaired *t* test. For analyzing the medians of overall survival between groups, we used the Mann-Whitney rank sum test. The statistical tests were performed using SigmaStat software (version 3.0.1, 1992-2003, SPSS, Chicago, IL).

## Results

### Clinical Features

There were no statistically significant associations seen between the clinical features and diagnosis ▮Table 2▮ (*P* > .05). The median age of patients with DLBCL at diagnosis was higher than for patients with BL. Fewer patients in the total group and in the BL and DLBCL groups had AIDS-related disease than non–AIDS-related disease. Advanced stage lymphoma (III/IV) was observed at similar frequencies in the total group and the 2 subgroups. At diagnosis, almost three fourths of all patients had a high serum level of lactate dehydrogenase; the proportion was similar in the BL and DLBCL groups. Risk stratification by the International Prognostic Index was fairly consistent across the total and BL and DLBCL groups. The median overall survival was short (≤15 months). The short overall survival may be due to a high percentage of AIDS-related lymphomas and nonuniform therapy across multiple institutions.

**❚Table 2❚**
**Clinical Features for Patients With BL and DLBCL***

| | All Cases (n = 55) | BL (n = 16) | DLBCL (n = 39) | P |
|---|---|---|---|---|
| Sex | | | | .213 |
| M | 37 (67) | 13 (81) | 24 (62) | |
| F | 18 (33) | 3 (19) | 15 (38) | |
| Age (y) | | | | .070 |
| Median | 50 | 44 | 54 | |
| Range | 3-90 | 3-79 | 4-90 | |
| AIDS status | | | | .126 |
| − | 34 (62) | 7 (44) | 27 (69) | |
| + | 21 (38) | 9 (56) | 12 (31) | |
| Stage | | | | .775 |
| I/II | 26 (47) | 7 (44) | 19 (49) | |
| III/IV | 29 (53) | 9 (56) | 20 (51) | |
| Extranodal sites | | | | .360 |
| <2 | 34 (62) | 8 (50) | 26 (67) | |
| ≥2 | 21 (38) | 8 (50) | 13 (33) | |
| Performance status | | | | .370 |
| Ambulatory | 36 (65) | 9 (56) | 27 (69) | |
| Nonambulatory | 19 (35) | 7 (44) | 12 (31) | |
| LDH level | | | | .370 |
| Normal | 12 (22) | 2 (13) | 10 (26) | |
| High | 39 (71) | 12 (75) | 27 (69) | |
| Unknown | 4 (7) | 2 (13) | 2 (5) | |
| IPI risk group | | | | 1.000 |
| Low (0-2) | 29 (53) | 8 (50) | 21 (54) | |
| High (3-5) | 26 (47) | 8 (50) | 18 (46) | |
| Overall survival (mo) | | | | .795 |
| Median | 15 | 13 | 15 | |
| Range | 0-152 | 0-152 | 0-136 | |

BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; IPI, International Prognostic Index; LDH, lactate dehydrogenase.
* Data are given as number (percentage) unless otherwise indicated.

## Morphologic Diagnosis

We morphologically diagnosed each case as DLBCL (centroblastic or immunoblastic subtypes) or BL (classic or variant subtype) according to traditional WHO criteria.[6] Each of the 4 lymphoma subtypes is illustrated ❚Image 1❚. The 2 BL subtypes are classic (Image 1A) and variant (Image 1B). DLBCLs are cytologically diverse and can be divided into 2 major morphologic variants,[6] the centroblastic variant (Image 1C) and the immunoblastic variant (Image 1D).

## Support of Morphologic Diagnosis

In addition to the morphologic diagnosis, the distinction between variant BL and DLBCL was supported by a high Ki-67 proliferation index (Ki-67 score, 2) and/or the presence of a c-*myc* translocation as determined by FISH in paraffin-embedded sections. Among the cases with technically adequate FISH signals, c-*myc* rearrangement was detected in 10 BL cases and none of 16 DLBCL cases tested.

## Immunohistochemical Expression

❚Image 2❚ shows the results of application of our panel of immunohistochemical markers to 4 representative morphologically diagnosed cases. For BL, GC protein expression was strongest, which is consistent with its hypothesized GC cell origin, whereas DLBCL showed a spectrum of stronger ABC expression.

## Hierarchical Cluster Analysis

❚Figure 1❚ gives the results of hierarchical clustering applied to 8 GC and ABC immunohistochemical markers for 16 BL and 39 DLBCL cases. Two major clusters were evident: the Burkitt and the DLBCL. There was a statistically significant association between the immunophenotypes of the Burkitt cluster and the DLBCL cluster with the corresponding morphologic diagnoses of BL and DLBCL (P < .001; Fisher exact test). Based on the hierarchical cluster analysis, the sensitivity of diagnosing BL was 0.81 and the specificity was 0.87 ❚Table 3❚.

## Contour-Frequency Plot Analysis

❚Figure 2❚ gives the contour-frequency plot for all 55 cases. Case-frequency contours also are given. By using receiver operating characteristic curve analysis, we maximized sensitivity and specificity by positioning a diagonal line across the frequency plot, creating a BL group and a DLBCL group. BL cases are located predominantly in the upper-left triangle of Figure 2, corresponding to high GC and low ABC scores. In contrast, DLBCL cases are located predominantly in the lower-right triangle, corresponding to high ABC and low GC scores. The BL group includes 15 BL and 7 DLBCL cases, and the DLBCL group includes 1 BL and 32 DLBCL cases. There was a statistically significant association between the BL group and the DLBCL group with their corresponding morphologic diagnoses of BL and DLBCL (P < .001; Fisher exact test). The sensitivity of diagnosing BL was 0.94 and the specificity of diagnosing BL was 0.82 ❚Table 4❚.

## Discussion

Considerable overlap exists between the morphologic features of DLBCL and BL. In particular, high-grade Burkitt-like lymphoma (BLL), described as a provisional entity in the revised European-American classification of lymphoid neoplasms[21] and as a subtype of BL in the WHO classification,[6,22] has morphologic characteristics intermediate between classic BL and DLBCL of the centroblastic or immunoblastic type. The Non-Hodgkin's Lymphoma Classification Project[23] found that distinguishing between BLL and DLBCL was extremely difficult.

Many studies have reported the presence of individual protein expression markers on BL and DLBCL such as the B-cell antigens CD19, CD20, and CD22[6,24] and the germinal center markers CD10 and bcl-6.[25-31] Activation-induced cytidine deaminase is highly expressed in BL but variably expressed in
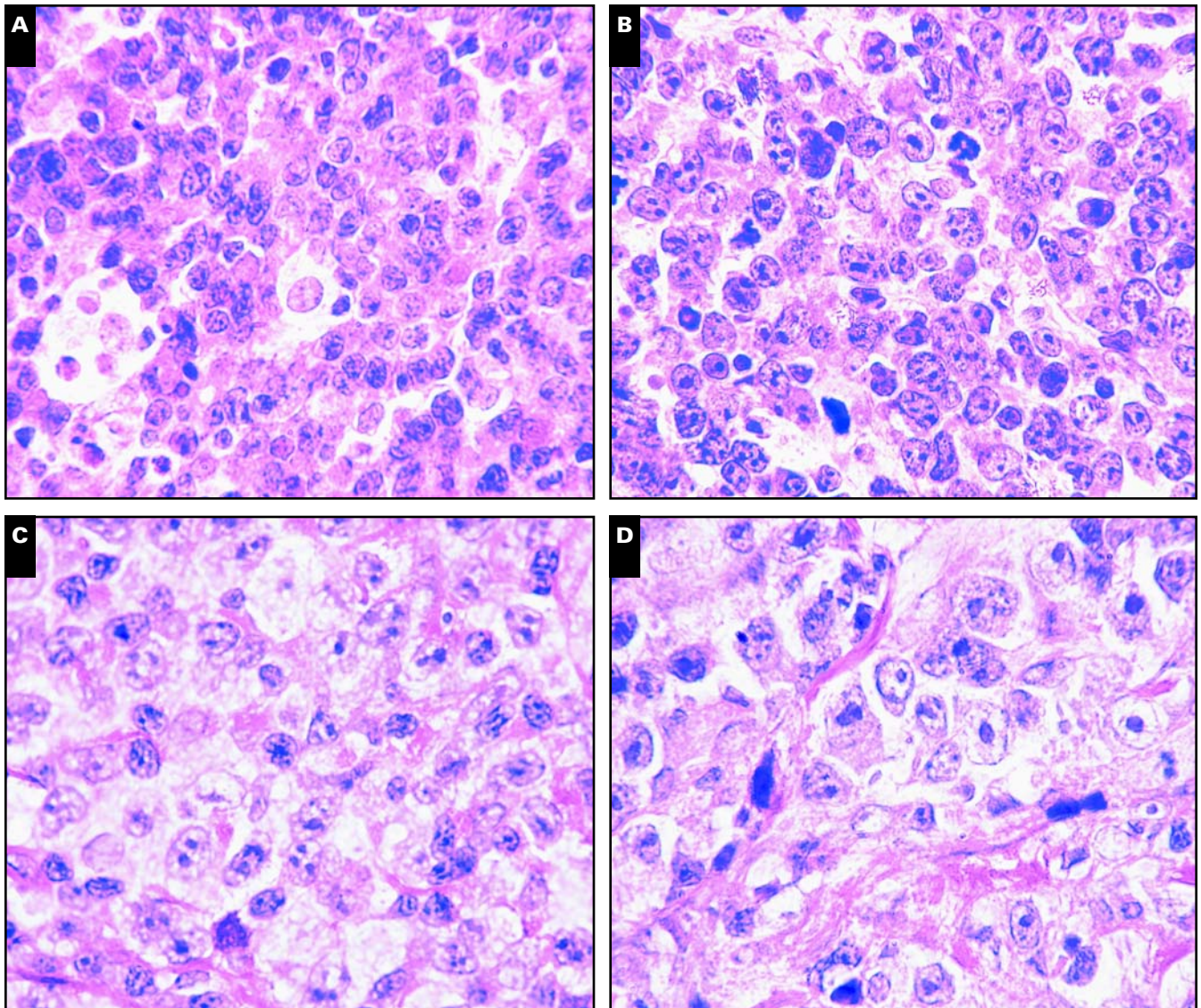
**❚Image 1❚ A**, Classic Burkitt lymphoma (BL) characterized by monomorphic, small to medium cells, arranged in a diffuse sheet and interspersed with tingible-body macrophages, giving the well-known "starry-sky" pattern.[6] The cytoplasm often contains lipid vacuoles. The nuclei are round and noncleaved, with clumped chromatin and multiple, centrally located nucleoli. The nuclei are similar in size to the accompanying macrophages (H&E, ×1,000). **B**, Variant BL, ie, atypical Burkitt or Burkitt-like lymphoma shows greater nuclear pleomorphism and more prominent but fewer nucleoli than classic BL (H&E, ×1,000). **C**, Diffuse large B-cell lymphoma (DLBCL), centroblastic variant, contains medium to large cells with a moderate amount of cytoplasm. The nuclei appear round to oval with 2 to 4 nucleoli (H&E, ×1,000). **D**, DLBCL, immunoblastic variant, consists of large cells with an oval vesicular nucleus and a single, centrally located nucleolus, plus abundant cytoplasm (immunoblasts) (H&E, ×1,000).

DLBCL.[32] In BL, nearly 100% of tumor cells express the Ki-67 proliferation marker.[6,21,24,33,34] To make a diagnosis of BLL, the WHO classification requires a high growth fraction of nearly 100%, similar to BL.[6] However, high Ki-67 expression is not unique to BL. In a study of 899 patients with non-Hodgkin lymphoma (including DLBCL), Miller et al[35] found that the Ki-67 index ranged from 4% to 95% (median, 50%). Of 41 diffuse large cell lymphomas, 34 had a Ki-67 proliferation index of more than 80%. In a study of pediatric BL and DLBCL, there was a significant difference in MIB-1 (Ki-67) expression (BL, 99% vs DLBCL, 56%; $P < .0001$).[29] In another pediatric study, Ki-67 was expressed uniformly in all 3 categories of BL, BLL, and DLBCL.[26] bcl-2 is a proto-oncogene product whose expression suppresses apoptosis. Expression of bcl-2 protein is positive in some DLBCL cases[6,26,28] and in a portion of BLL cases.[26] CD138 (syndecan), a marker associated with antigen-stimulated B cells, is expressed in some cases of DLBCL of the ABC type.[36] Because MUM1, a member of the interferon
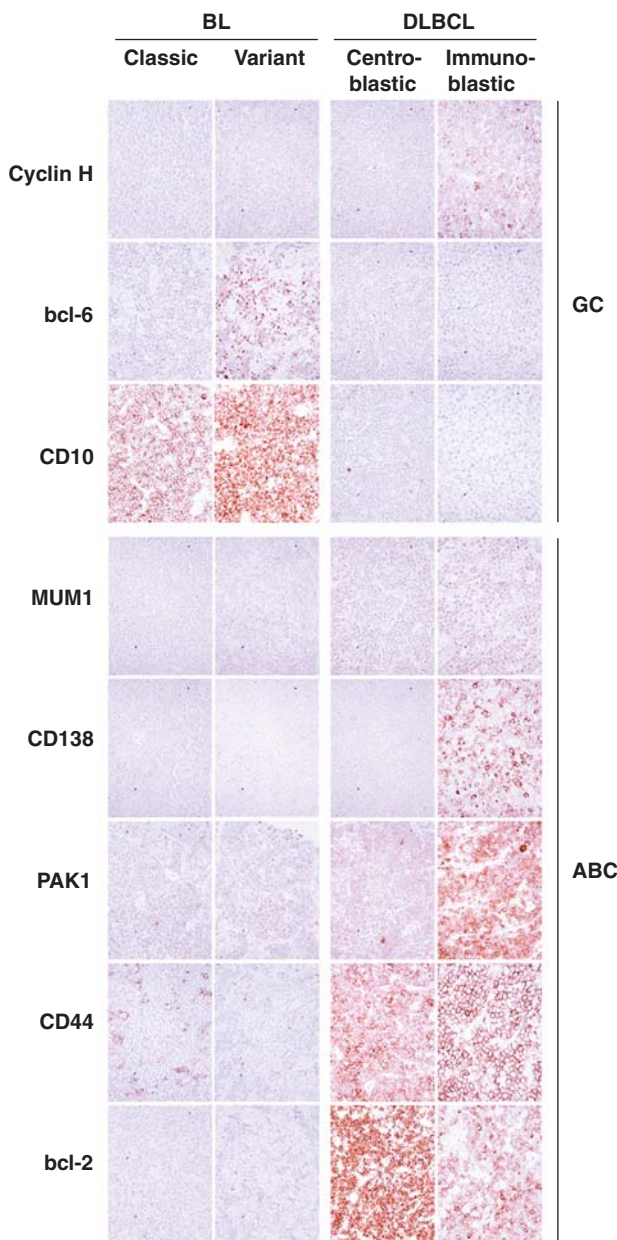
**Image 2** Panel of immunohistochemical markers applied to 4 representative morphologically diagnosed cases. The 2 left columns represent classic and variant Burkitt lymphoma; the 2 right columns represent centroblastic and immunoblastic diffuse large B-cell lymphoma (×500). ABC, activated B-cell phenotype; GC, germinal center phenotype.
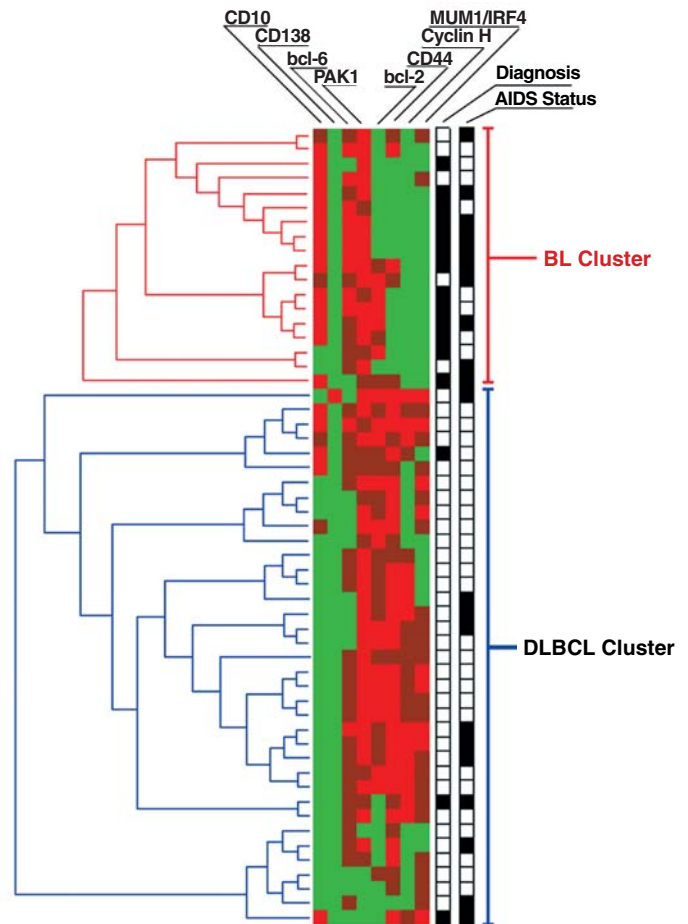


**Figure 1** Hierarchical clustering applied to 8 germinal center and activated B-cell immunohistochemical markers for 16 cases of Burkitt lymphoma (BL) and 39 of diffuse large B-cell lymphoma (DLBCL). Two major clusters are evident: the Burkitt cluster containing 13 BL and 5 DLBCL cases (top, red dendrogram) and the DLBCL cluster containing 3 BL and 34 DLBCL cases (bottom, blue dendrogram). Strong staining is indicated by red, weak staining by brown, and absence of staining by green. Diagnosis is indicated by a black square for BL and white square for DLBCL. AIDS status is indicated by a black square for AIDS and white square for non-AIDS.

**Table 3**
**Hierarchical Cluster Groups Correlate With Morphologic Diagnosis**[*]

| | Morphologic Diagnosis | |
| --- | --- | --- |
| **Cluster** | **BL** | **DLBCL** |
| BL | 13 | 5 |
| DLBCL | 3 | 34 |

BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma.
[*] Sensitivity of diagnosing BL, 0.81; specificity of diagnosing BL, 0.87.

regulatory factor family of transcription factors,[37-39] is expressed during the ultimate step of GC B-cell maturation and development into plasma cells,[40] it has been used as a marker of non-GC B cells. It has controversial value as a single phenotypic predictor of survival in DLBCL.[39,41] CD44 is composed of multiple isoforms that participate in lymphocyte migration, homing, and activation.[42,43] Its expression in DLBCL predicts poor survival.[44-47] Overall, no single antibody can reliably distinguish BL from DLBCL.

In contrast, only a few studies have used multiple markers to differentiate BL from DLBCL. Compared with DLBCL,
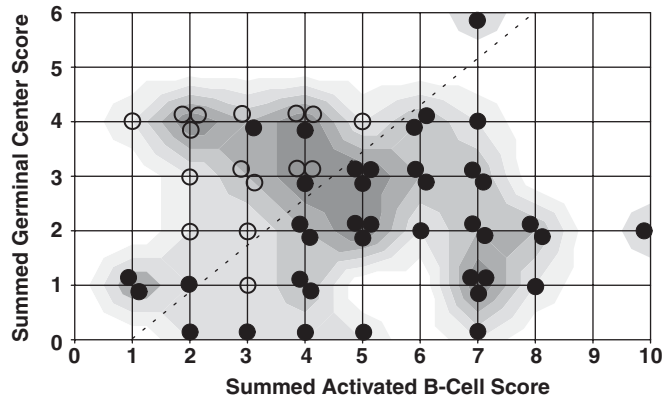
**Figure 2** Contour-frequency plot for 55 cases of Burkitt lymphoma and diffuse large B-cell lymphoma. The y-axis represents the sum of the individual immunohistochemical marker scores for the germinal center (GC) phenotype, bcl-6 + CD10 + cyclin H. The x-axis represents the sum of the individual immunohistochemical marker scores for the activated B-cell (ABC) phenotype, MUM1 + CD138 + PAK1 + CD44 + bcl-2. Each lymphoma case is indicated by a unique (x, y) coordinate data point, which is determined by the ABC and GC scores. In the case-frequency contours (gray), the density of gray is proportional to the number of cases. Cases with identical coordinates have been slightly offset to display all data points. Open circles, BL cases; filled circles, DLBCL cases.

**Table 4**

**GC/ABC Contour Plot Groups Correlate With Morphologic Diagnosis***

| | Morphologic Diagnosis | |
|---|---|---|
| Group | BL | DLBCL |
| BL | 15 | 7 |
| DLBCL | 1 | 32 |

ABC, activated B cell; BL, Burkitt lymphoma; DLBCL, diffuse large B-cell lymphoma; GC, germinal center.
* Sensitivity of diagnosing BL, 0.94; specificity of diagnosing BL, 0.82.

BLL has a higher proliferation rate (Ki-67), greater expression of CD10 (GC marker), decreased expression of bcl-2 (ABC marker), and consistently absent expression of CD44.[48] A combined panel characteristic for BL and "helpful" in distinguishing BL from DLBCL includes positive CD10, absence of bcl-2 protein, and an extremely high MIB-1 (Ki-67) proliferative index.[7] An immunohistochemical panel consisting of c-myc, bcl-2, and bcl-6 applied to 41 pediatric cases with B-cell lymphoma revealed that most childhood BLLs but not BLs express bcl-6 and that most large B-cell lymphomas, only a small portion of BLLs, and no BLs express bcl-2.[26] Frost et al[29] found a statistically significant difference in the protein expression of c-myc, bcl-2, and MIB-1 (Ki-67). Specifically, strong positive expression of c-myc, a MIB-1 proliferative index of more than 99%, and absence of bcl-2 was highly predictive of a BL diagnosis. The expression of both CD10 and bcl-6 was highly variable for differentiating BL from DLBCL.[31]

The difficulty distinguishing BL and DLBCL in a subset of cases has led to ancillary molecular studies. All cases of BL have a *myc* translocation from chromosome 8 to the immunoglobulin heavy chain region on chromosome 14 (most common) or to light chain loci on chromosome 2 or 22.[6] However, the *myc* translocations are not completely specific to BL. In fact, a significant subset of DLBCL cases show c-*myc* translocations ranging from 10% to 25%.[7-10] Molecular assays are not always available, and if they are, there is an increase in cost and turnaround-time. Because 29 (53%) of 55 cases were technically inadequate for FISH analysis, we do not believe that FISH analysis of *myc* can be relied on as the "gold standard" for diagnosing BL in paraffin-embedded tissue samples. This is another reason why a search for additional immunophenotypic markers to separate BL from DLBCL in tissue sections is needed. Two recent studies used gene expression microarrays to differentiate BL from DLBCL with some success.[49,50] Although gene expression arrays are promising for diagnosis and subclassification of lymphomas, they are expensive and not widely available.

We used 2 methods to separate BL from DLBCL using the protein expression of 8 immunohistochemical markers on 16 BL and 39 DLBCL cases. The 8 protein markers included 3 GC and 5 ABC antibodies.

In the first method, we applied an unsupervised analysis using a freely available hierarchical cluster algorithm originally designed for gene expression arrays.[18] We ran the cluster algorithm on all 8 individual protein markers without a priori knowledge of the morphologic diagnosis. The algorithm yielded 2 major clusters (Figure 1), 1 containing primarily BL cases (13 BL and 5 DLBCL) and the other, primarily DLBCL cases (3 BL and 34 DLBCL). The sensitivity of diagnosing BL was 0.81 and the specificity of diagnosing BL was 0.87. As expected, the GC markers, CD10 and bcl-6, were expressed strongly in the BL cluster and much less so in the DLBCL cluster. Unexpectedly, the third GC marker, cyclin H, was completely absent in the BL cluster and strongly expressed in the DLBCL cluster. The ABC marker bcl-2 was nonspecifically expressed in both clusters (44% in the BL cluster and 78% in the DLBCL cluster). With respect to Figure 1, the BL cluster, of the 7 bcl-2+ cases with the morphologic diagnosis of BL, 3 were AIDS-related. Expression of bcl-2 protein has been detected by others in a minority (up to 15% of cases) of sporadic BL and BLL.[7,48,51] The ABC markers CD44 and MUM1 were, as expected, primarily absent in the BL cluster and strongly positive in the DLBCL cluster. The ABC markers

PAK1 and CD138 did not contribute to cluster separation: PAK1 was strongly positive for both clusters, and CD138 expression was absent except in 1 case.

In the second method, we separately summed the immunohistochemical staining scores of the 3 GC markers and the 5 ABC markers for each case, reducing an 8-dimensional problem to 2 dimensions, GC score and ABC score. Plotting the ABC-GC summation score for each case as an x-y data point on a 2D grid of GC (y-axis) vs ABC (x-axis) yielded an easily interpretable cluster diagram (Figure 2). By using the morphologic diagnosis (supported by c-myc and Ki-67 data), as a priori knowledge, we applied a diagonal dividing line to this 2D plot to create a BL group and a DLBCL group. Sensitivity and specificity were maximized by receiver operating characteristic curve analysis by incrementally moving the dividing line. This method of separating data points into groups based on the a priori knowledge of the morphologic diagnosis is commonly referred to as *supervised* analysis. As expected, we found that the BL cases expressed a predominantly GC phenotype and the DLBCL cases a spectrum of lower GC and increased ABC phenotype. It is interesting to note that there is a continuum of ABC-GC expression from the BL group to the DLBCL group with no distinct separation between the 2 groups.

Even though this is a pilot study with a limited number of cases, we were able to confirm and extend the value of multi-parameter gene expression using immunohistochemical markers for the diagnosis of BL vs DLBCL.

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