bjh research paper

Diagnosis of Burkitt lymphoma using an algorithmic approach – applicable in both resource-poor and resource-rich countries

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Received 19 May 2011; accepted for publication 23 May 2011

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Summary

Distinguishing Burkitt lymphoma (BL) from B cell lymphoma, unclassifiable with features intermediate between diffuse large B-cell lymphoma (DLBCL) and BL (DLBCL/BL), and DLBCL is challenging. We propose an immunohistochemistry and fluorescent *in situ* hybridization (FISH) based scoring system that is employed in three phases – Phase 1 (morphology with CD10 and BCL2 immunostains), Phase 2 (CD38, CD44 and Ki-67 immunostains) and Phase 3 (FISH on paraffin sections for *MYC*, *BCL2*, *BCL6* and *immunoglobulin family* genes). The system was evaluated on 252 aggressive B-cell lymphomas from Europe and from sub-Saharan Africa. Using the algorithm, we determined a specific diagnosis of BL or not-BL in 82%, 92% and 95% cases at Phases 1, 2 and 3, respectively. In 3·4% cases, the algorithm was not completely applicable due to technical reasons. Overall, this approach led to a specific diagnosis of BL in 122 cases and to a specific diagnosis of either DLBCL or DLBCL/BL in 94% of cases that were not diagnosed as BL. We also evaluated the scoring system on 27 cases of BL confirmed on gene expression/microRNA expression profiling. Phase 1 of our scoring system led to a diagnosis of BL in 100% of these cases.

Keywords: lymphoma, diagnostic haematology, immunophenotyping.

Burkitt lymphoma (BL) is among the most studied human malignancies. While the diagnosis of BL in the paediatric age group is more straightforward, identifying a reliable subtype of adult aggressive B-cell lymphomas remains a challenge (Dave *et al*, 2006; Hummel *et al*, 2006). Improvements in therapeutic options for adult aggressive B-cell lymphomas make distinction of BL from diffuse large B-cell lymphoma (DLBCL) and other lymphomas extremely critical (Dave *et al*, 2006; Hummel

First published online 1 July 2011
doi:10.1111/j.1365-2141.2011.08771.x
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et al, 2006; Mead *et al*, 2008). In the World Health Organization (WHO) classification, BL is defined as a B-cell lymphoma with a short doubling time often presenting at extranodal sites or as acute leukaemia. It is composed characteristically of monomorphic cells of medium size, with finely clumped and dispersed chromatin, and with multiple basophilic paracentrally located nucleoli. The cells display some degree of cohesion, with abundant mitoses and apoptoses. However, a proportion of cases may show variation in cell, nuclear and nucleolar size, and some have more prominent plasmacytoid features (often seen in the immune deficiency setting). BL has a typical immunophenotype – strong immunoglobulin expression; expression of B-cell antigens and germinal centre (GC) markers, absence of BCL2 expression; and a near 100% Ki-67 expression. However, about 20% cases or less show very weak and heterogeneous expression of BCL2 and rare cases can be CD10 negative. Furthermore, nearly one-third of cases with a molecular signature of BL has <95% Ki-67 expression (rare cases <90% proliferation). Typically, BL harbours the *MYC-IGH* translocation, t(8;14)(q24;q32), and less commonly the translocation partner of the *MYC* gene is one of the immunoglobulin light chain genes (22q11 or 2p12). However, up to 10% of BL lack demonstrable *MYC*-translocation. Typically, *MYC-immunoglobulin family (IG)* translocation occurs as the sole abnormality (*MYC*-simple karyotype) in BL. Despite such well characterized features, none of the above parameters can be singly used for the diagnosis of BL. The WHO classification suggests that a combination of several diagnostic techniques is necessary for the diagnosis (Leoncini *et al*, 2008; Raphael *et al*, 1991).

BL needs to be distinguished from the subset of lymphomas in the newly identified category of B cell lymphoma, unclassifiable with features intermediate between DLBCL and BL (DLBCL/BL), and which has an extremely poor prognosis. The cell size is usually medium or slightly larger. The tumour cells show variation in cell, nuclear and nucleolar size. The immunophenotype is variable with a higher proportion of cases expressing BCL2. Though *MYC-IG* translocation occurs in a proportion of DLBCL/BL, the more characteristic feature is presence of *MYC* translocation with non-*IG* partners. Furthermore, cases of DLBCL/BL with *MYC* translocation also carry additional chromosomal translocations or other cytogenetic abnormalities (*MYC*-complex karyotype), and it includes cases of the so-called double-hit lymphomas (cases with *MYC* and *BCL2* translocations or *MYC* and *BCL6* translocations). On array comparative genomic hybridization (CGH) the *MYC*-complex karyotype is characterized by six or more abnormalities in addition to *MYC* translocation (Dave *et al*, 2006; Hummel *et al*, 2006; Leoncini *et al*, 2008; Kluin *et al*, 2008; Snuderl *et al*, 2010; Barrans *et al*, 2010).

It should be remembered that a proportion of DLBCL cases may also have some of the individual characteristics of BL, such as lack of BCL2 expression, >95% Ki-67 expression or *MYC* translocation. Almost all cases of DLBCL with translocations involving the 8q24 locus (*MYC* gene) have additional cytogenetic abnormalities (Ladanyi *et al*, 1991).

Gene expression studies suggest the presence of distinct molecular signatures in BL and DLBCL samples. BL shows high level of expression of MYC target genes and a subgroup of GC B-cell genes, and a low level of expression of major-histocompatibility-complex class I and nuclear factor (NF)-κB target genes. Though gene expression signatures appear to be the 'gold standard' in distinguishing BL, DLBCL/BL and DLBCL, complexities associated with its interpretation make them unsuitable for routine diagnosis even in the best of centres in the world. Evaluating the expression (by immunohistochemistry) of some of the specific differentially expressed molecules brought to light by gene expression studies can be an alternative to conventional gene expression studies. Immunohistochemical evaluation of some of these markers is well established. They include CD40, MUM1 and CD44 (NFκB pathway targets) and CD38 and GCET2 (germinal centre genes) (Dave *et al* 2006). Although gene expression studies showed that BL did not express *MUM1* other studies have suggested that MUM1 is expressed in a proportion of BL (Chuang *et al*, 2007). On an immunohistochemistry platform, CD38 and CD44 immunostains have been documented to distinguish *MYC* translocation-positive and negative lymphomas (Rodig *et al*, 2008).

Though robust strategies to distinguish true BL, DLBCL/BL and DLBCL are essential, from a practical standpoint it would be more beneficial to focus on two categories: true BL and others, given that the prognosis for the DLBCL/BL is uniformly poor (Tomita *et al*, 2009; Niitsu *et al*, 2009; Johnson *et al*, 2009; Keller *et al*, 2008). Furthermore, these strategies should be applicable in those geographic areas of the world where BL is more common: Africa and rest of the developing world.

Material and methods

Proposal of an algorithm/scoring system for diagnosis of BL

The algorithm is for use in aggressive lymphomas expressing CD20 and would typically be advocated for cases with morphology in the range of BL and DLBCL. It would be important not to include B lymphoblastic lymphomas and blastoid variants of mantle cell lymphoma before employing this algorithm. In lymphomas composed of monomorphic infiltrate of medium sized lymphoid cells with fine chromatin and lack of conspicuous nucleoli, absence of TdT and/or cyclin D1 expression is essential before the scoring system is employed. Cases would be evaluated by morphology and a small panel of immunostains (Phase 1). Those cases not resolved at this stage would be investigated by a larger panel of immunostains (Phase 2). Those that remain unresolved would then need fluorescent *in situ* hybridization (FISH) analysis (Phase 3) (Fig 1).

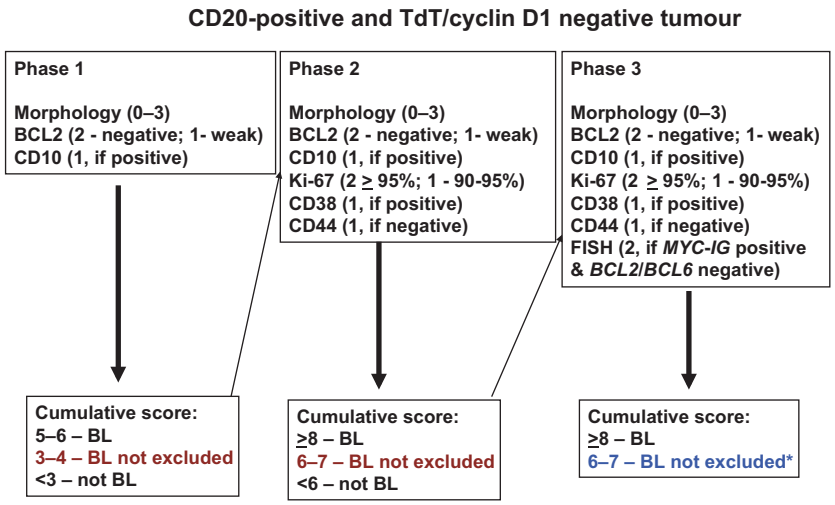

Fig 1. Algorithm/scoring system for Burkitt lymphoma. The algorithm/scoring system to be used in three phases in aggressive B-cell lymphomas where the morphology is in the range of BL, DLBCL/BL and DLBCL. Critically, B lymphoblastic lymphoma and blastoid mantle cell lymphoma should be excluded before employing this algorithm.

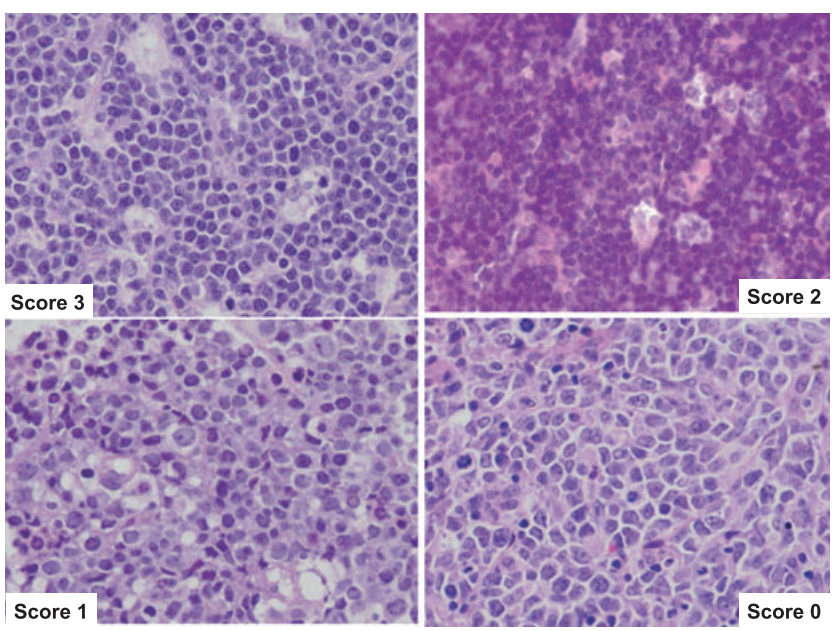

Fig 2. Morphological score. Images [Haemotoxylin & Eosin (H&E) ×400] from cases where the morphology was scored as score 3 (features of BL), 2 (suspicious, but not diagnostic of BL, due to technical reasons), 1 (features overlapping between BL and DLBCL) and 0 (features of DLBCL).

Scoring for Phase 1.

- Morphology (Fig 2): typical BL – 3; morphology consistent but not diagnostic of BL due to technical reasons – 2; overlapping features between BL and DLBCL – 1; DLBCL – 0.
- BCL2: Absent – 2; weak – 1; moderate to strong – 0.
- CD10: Positive (irrespective of intensity) – 1; completely negative – 0.

Cumulative scores at Phase 1: Score 5–6: diagnosis of BL; Score 3–4: BL not excluded (proceed to Phase 2); Score <3: not BL.

Additional investigations and scoring for Phase 2.

- Ki-67: >95% – 2; 90–95% – 1; <90% – 0.
- CD38: Positive – 1; negative – 0.
- CD44: Negative – 1; positive – 0.

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Cumulative scores at Phase 2: Score ≥8: diagnosis of BL; Score 6–7: BL not excluded (proceed to Phase 3); Score <6: not BL.

Additional investigations and scoring for Phase 3. Interphase FISH: positive for rearrangement of *MYC* and one of the *IG* genes, and negative for *BCL2* and *BCL6* rearrangements – 2; others – 0.

Cumulative scores at Phase 3: Score ≥8: diagnosis of BL; Score 6–7: BL not excluded (the diagnostic impact of each of the different parameters need to be assessed; complete karyotype and array CGH would be useful).

Understandably, directly employing the 'Phase 3' part of the algorithm would have the best ability to identify true BL. However, Phases 1 and 2 of the algorithm would enable precise diagnosis of BL in laboratories with relatively less infrastructure and expertise, and in countries with limited resources.

Evaluation of the algorithm

Two hundred and fifty-two (252) cases of aggressive B-cell lymphomas in which all attempts had been made to arrive at a specific diagnosis based on the current WHO classification were collated for the study. One hundred and twenty cases represented either routine diagnostic cases of Hammersmith Hospital, London, UK or represented those cases that had been evaluated for other lymphoma studies at the Hammersmith Hospital. The remaining cases (132) were from sub-Saharan African countries, and the work-up was undertaken either at University of Siena, Italy (114 cases) or University of Paris, France (18 cases). The cases included both children and adult patients. Twenty-seven patients were documented to be human immunodeficiency virus (HIV) positive (11 cases of BL, four cases classified as BL not excluded and 12 cases of DLBCL). Information on Epstein-Barr virus (EBV) association [expression of EBV-encoded small RNA (EBER) by *in-situ* hybridization] was available in 57 cases and 31 were recorded to be positive (20 of 27 BL, three of eight cases classified as BL not excluded, two of four DLBCL/BL and 4 of 18 DLBCL). All cases had been reviewed independently by three haematopathologists.

Results of the following immunostains that had been recorded at the time of diagnosis were collated to evaluate the newly-devised BL algorithm – CD10 (247 cases), BCL2 (252 cases), CD38 (148 cases), CD44 (128 cases) and Ki-67 (168 cases). FISH analysis for breakpoints on *MYC*, *BCL2*, *BCL6* and *IGH* genes had been undertaken on paraffin sections in 129 cases. Cases that had *MYC* rearrangement without *IGH* rearrangement had been further analysed for *IGK* and *IGL* rearrangements by FISH. Based on the algorithm, cases were classified into BL, BL not excluded (BLNE) and not-BL categories. These were compared with the diagnosis previously made.

Further evaluation of the algorithm

The algorithm was further evaluated on two independent sets of BL; one on which the gene expression analysis had confirmed an expression pattern of BL (12 cases) (Piccaluga *et al*, 2011) and the other on which the microRNA expression had confirmed an expression pattern of BL (15 cases) (Lenze *et al*, 2011). The authors of the current study had contributed the above cases for studies on gene and microRNA expression.

Results

In 206 of 252 cases (81·7%), Phase 1 of the scoring system categorized cases either as BL (105 cases) or not-BL (99 cases). Among the remaining cases, two labelled as BLNE could not evaluated by Phase 2 of the scoring system due to failed Ki-67 immunostain that could not be repeated due to paucity of available tissue. These two cases however had rearrangements of *MYC* and *IGH* genes without involvement of *BCL6* and *BCL2* genes, and were diagnosed as BL. The remaining 46 cases proceeded to Phase 2 of the scoring system, at which 27 cases (10·7%) were categorized as BL (10 cases) or not-BL (17 cases). In nine of the remaining 19 cases, Phase 3 of the scoring system could not be applied as FISH results were not available due to technical reasons (paucity or the technical quality of the tissue available) and these were categorized as BLNE. Phase 3 of the scoring system was employed in 10 cases; a diagnosis of BL was made in seven cases (2·8%) and the other three were categorized as BLNE (Fig 3).

Collectively, the algorithm yielded a diagnostic category in 240 cases (95·2%). Furthermore, morphology supported by three immunostains (CD20, CD10 and BCL2; Phase 1) categorized *c.* 82% of aggressive B-cell lymphomas as those that are BL and those that are not. This improved to *c.* 92% by addition of three more immunostains (CD38, CD44 and Ki-67; Phase 2). FISH analysis (Phase 3) on paraffin sections improved the diagnostic ability by an additional 3%. However, FISH was able to resolve the diagnosis in 70% of cases where

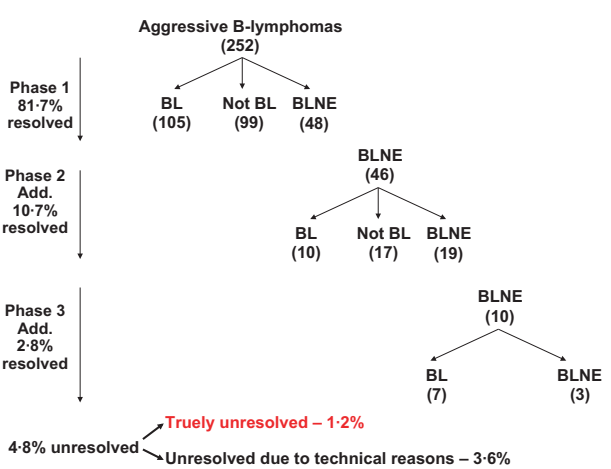

Fig 3. Application of the algorithm in 252 aggressive B-cell lymphomas. Diagnosis was resolved in 81·7%, 92·4% and 95·2% of cases at Phases 1, 2 and 3, respectively. BL, Burkitt lymphoma; BLNE, BL not excluded.

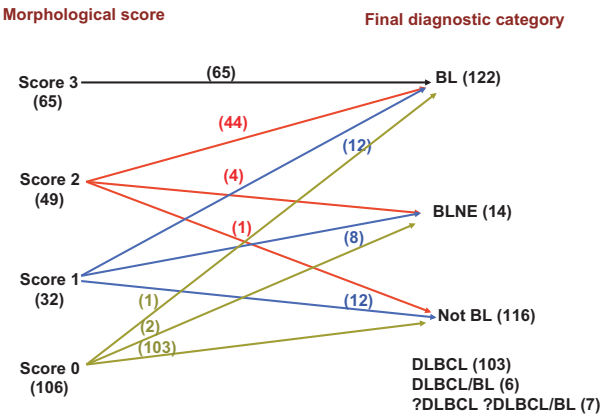

Fig 4. Correlation of the initial morphological score with the final diagnostic score. The figure demonstrates how the initial morphological score correlated with the final diagnostic score after immunohistochemistry and FISH analysis. The cases had however been evaluated independently by three experienced hematopathologists and the H&E sections had been prepared in Europe. BL, Burkitt lymphoma; BLNE, BL not excluded; DLBCL, diffuse large B-cell lymphoma.

Phase 3 of the algorithm was required. The algorithm was unable to offer a specific diagnosis in only 1·2% cases, and a complete karyotype with or without array CGH, would probably help in arriving at a precise diagnosis. Due to technical reasons, the algorithm could be not fully applied in nine cases (3·4%) (Fig 3).

Among the 122 cases diagnosed as BL, the morphology scores were 3, 2, 1 and 0 among 65, 44, 12 and 1 case(s), respectively. In one case (the case with score 0), the initial diagnosis was DLBCL. Among 14 cases that were categorized as 'BLNE', the morphology scores were 2, 1 and 0 in 4, 8 and 2 cases, respectively. Among the 116 cases categorized as not-BL, the morphology scores were 2, 1 and 0 in 1, 12 and 103 cases, respectively (Fig 4).

The final diagnosis in cases categorized as not-BL (116) following incorporation of all parameters was: DLBCL in 103, DLBCL/BL in 6 (including one case of double-hit lymphoma), and in seven cases a decision between DLBCL and DLBCL/BL could not be made (Fig 4).

Among the cases categorized as BL, six cases (5%) showed BCL2 expression and three cases (2·5%) lacked CD10 expression. Ki-67 expression was ≤95% in 9·3% cases. Among cases that were considered BLNE, 50% showed BCL2 expression, one case (7%) lacked CD10 expression and 73% had ≤95% Ki-67 expression. Results of CD38 and CD44 immunostains were available in 125 cases. The $CD38^-/CD44^+$ phenotype seen in 47 cases was completely restricted to cases categorized as not-BL, and in 41 of these 47 cases, categorization had been performed independent of the CD38 and CD44 results (Phase 1 of the scoring system) (Table I). The $CD38^+/CD44^-$ phenotype was seen in 48 cases, and the phenotype was seen in 73%, 75% and 19% of cases categorized as BL, BLNE and not-BL, respectively. Twenty cases were negative for both CD38 and CD44, and 10 cases were positive for both.

Table I. Results of immunohistochemistry and FISH in different diagnostic categories.

Immunohistochemistry	BL (%)	BLNE (%)	Not BL (%)
BCL2 expression	5	50	72
Absence of CD10 expression	2·5	7	67
Ki-67 ≤95%	9·3	73	94
$CD38^-/CD44^+$ phenotype	0	0	59
Presence of rearrangements of *MYC* and *IG* genes in the absence of *BCL2* or *BCL6* rearrangements	90	67	5

BL, Burkitt lymphoma; BLNE, BL not excluded.

Among the 48 cases categorized as BL with available FISH results, 43 (90%) had rearrangements of *MYC* and *IGH* genes, while the remaining 10% (five cases) lacked evidence of *MYC* or *IGH* rearrangements on FISH analysis. There was no evidence of *BCL2* or *BCL6* rearrangements in these cases. Of the six cases categorized as BLNE that had FISH results available, four demonstrated rearrangements of *MYC* and *IGH* (67%). Four of the 74 (5%) cases categorized as not-BL had rearrangements of *MYC* and *IG* genes (three involving *IGH* and one involving *IGL*) in the absence of *BCL2* or *BCL6* rearrangements (Table I). In addition, one of the DLBCLs had rearrangement of *MYC* in the absence of a rearranged *IG* gene, and one of the four cases categorized as DLBCL/BL, had rearrangements of *MYC*, *BCL2* and *IGH* genes ('double-hit' lymphoma).

Among the 116 cases that were not BL, we were able to make a specific diagnosis of DLBCL in 103 cases and a diagnosis of DLBCL/BL in six cases (94%).

Validation of the algorithm on cases with gene and microRNA expression pattern of BL

Application of the algorithm to a set of 12 cases of BL confirmed on gene expression analysis showed scores of 5 (in five cases) and 6 (in seven cases) in the Phase 1 of the scoring system. Similarly, application of the algorithm to a set of 15 cases of BL confirmed on microRNA expression analysis showed scores of 5 (in seven cases) and 6 (in eight cases) in the Phase 1 of the scoring system.

Discussion

BL occurs in three clinical/epidemiological scenarios – endemic BL seen in equatorial Africa, Papua and New Guinea; sporadic BL seen in rest of the world; and immunodeficiency-associated BL seen in association with HIV or acquired immunodeficiency syndrome (AIDS) (Leoncini *et al*, 2008). Most of endemic-BL, and the majority of other BL occur in

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locations where the required infrastructure and technical expertise are not currently available, and may not be available in the near future. This aspect makes it pertinent to construct a diagnostic algorithm that would help making a reliable diagnosis of BL with greater ease and with lesser resources. Such a systematic approach is also relevant in the setting of developed countries, as none of the parameters currently used in the diagnostic evaluation can clearly distinguish the entities of BL, DLBCL/BL and DLBCL on an individual basis. Furthermore, the gene expression analysis that highlighted the issue of overlap between these entities is difficult, if not impossible, to be introduced for routine diagnostic usage even among the best of the institutions.

The scoring system-based algorithm we have suggested resulted in clear distinction between BL and cases that were not BL in 82% with use of only three antibodies (CD20, CD10 and BCL2). In a further 27 cases of BL with gene expression and microRNA expression profiles, the same three antibodies documented a diagnosis of BL in 100% of the cases. These three antibodies are relatively easy to use and can be employed in laboratories with limited experience in immunohistochemistry. This would be an ideal panel to initiate immunohistochemistry in centres that do not have the facility. We hope that this small panel of antibodies would make a great impact in the setting of developing countries especially in Africa.

The percentage of cells expressing Ki-67 is indeed a critical criterion for the diagnosis BL. However, as the cut-off for the distinction between BL and other aggressive B-cell lymphomas is either 95% or 90%, minor technical inadequacies or suboptimal tissue fixation could easily contribute towards underestimation of the proliferation to <90%. Hence, optimal tissue fixation and other technical aspects related to immunohistochemistry are of paramount importance. Due to these attributes Ki-67 immunostaining does not have a high inter-laboratory reproducibility. In the study by the Lunenburg Lymphoma Biomarker Consortium, which validated commonly used prognostic markers in DLBCL, variations in laboratory techniques and scoring contributed to lower agreement rates (53–58%) for BCL6 and Ki-67 immunostains. The participating centres were predominantly from the resource-rich countries (Keller *et al*, 2008). Accounting for such potential problems, the percentage of Ki-67 positive cells was introduced at Phase 2. BCL6 immunostain was not included in the algorithm based on the reported poor inter-laboratory reproducibility (de Jong *et al*, 2009).

Relatively recently, the expression of CD38 and CD44 has been shown to distinguish *MYC* translocation positive and negative lymphomas (Rodig *et al*, 2008). Given that experience with these antibodies is relatively limited; we introduced these antibodies in Phase 2 of the algorithm/scoring system. The most impressive distinction was the exclusion of the diagnosis of BL among cases that expressed CD44 in the absence of CD38. We have demonstrated that, by using a panel of six antibodies – CD20, CD10, BCL2, Ki-67, CD38 and CD44, one can accurately diagnose >92% of aggressive B-cell lymphomas and identify specific subsets within it.

In a small minority of cases where immunohistochemistry does not resolve the diagnosis, FISH analysis on paraffin sections for *MYC*, *BCL2*, *BCL6* and *IG* genes results in a specific diagnosis. It should be emphasized that a small minority of DLBCL (4·4% in this series) will harbour *MYC* rearrangements with or without a concomitant *IG* gene rearrangement and rearrangements involving *BCL2* or *BCL6* gene. The proportion of MYC rearranged cases varies depending on the inclusion/exclusion of cases of DLBCL/BL and cases of double/triple hit lymphomas. Presence of *MYC* rearrangement should not preclude from making a diagnosis of DLBCL. Similarly *c*. 10% of BL lack demonstrable *MYC-IG* translocations, and alternative pathogenetic mechanism(s) involving altered microRNA expression may be operational in such cases (Barrans *et al*, 2010; van Rijk *et al*, 2008; Leucci *et al*, 2008). Hence, a systematic/algorithmic approach to diagnosis is essential.

We have provided a simple schema to diagnose BL using a scoring system that is approached in three Phases. Phase 1 relies on morphology and a very small panel of immunostains that should be practicable in any part of the world and would lead to diagnosis of BL in >80% cases. Phase 2 utilizes three additional immunostains that are easy to apply and improves the diagnostic ability to >90%. In Phase 3, FISH analysis on paraffin sections is employed to resolve the diagnosis in virtually all cases. The introduction of FISH analysis only in Phase 3 of the scoring system also reduces costs and improves the turnaround time (Snuderl *et al*, 2010). It also makes the scoring system applicable in most parts of the world where FISH is not readily available or the costs associated with it discourage pathologists to employ the technique routinely.

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