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REVIEW ARTICLE

When and how to test for *C-MYC* in aggressive B cell lymphomas

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Abstract *C-MYC* (*MYC*) is a regulator gene that plays an important role in cell cycle progression, apoptosis, and cellular transformation. It is believed to regulate expression of up to 15 % of all human genes. In recent years, *C-MYC* has been extensively studied in blastic B cell lymphomas, and currently *C-MYC* is regarded not only as a diagnostic tool, but also a promising prognostic biomarker. *C-MYC* testing is therefore no longer simply a confirmational analysis for Burkitt's lymphoma, but also provides important information on prognosis and risk stratification of diffuse large B cell lymphoma patients. This review outlines our approach to integrate this new role of *C-MYC* in a convenient way in routine diagnostics and discusses the application of different techniques such as immunohistochemistry and FISH for *C-MYC* testing.

Keywords c-myc · *C-MYC* · DLBCL · Burkitt lymphoma · Immunohistochemistry · FISH · Prognosis · Double-hit score

Introduction

The *C-MYC* gene was identified more than 20 years ago as the cellular homolog of the retroviral *v-myc* oncogene [1–3]. Since then, its protein function, expression, and the epidemiology of its gene alterations have been intensively studied. *C-MYC* has been shown to be a regulator gene that codes for a transcription factor, a multifunctional nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis, and cellular transformation, and is believed to regulate expression of up to 15 % of all human genes [4]. Recent work suggests that c-myc is a TATA-Box-binding factor that regulates all genes transcribed by RNA-polymerase 2, meaning that c-myc is acting as a transcriptional amplifier rather than as a transcription factor per se [5]. Importantly, *C-MYC* plays a major role in the pathogenesis of malignant neoplasms, particularly lymphomas, and its gene is recurrently deregulated either by rearrangements, amplifications, or mutations [4]. Over 90 % of Burkitt's lymphomas (BL) carry a t(8;14) or, less commonly, a t(2;8), t(8;22), or t(3;8) [6, 7], juxtaposing the *C-MYC* gene to the immunoglobulin heavy chain, or the light chain gene. However, 5–14 % of diffuse large B cell lymphomas (DLBCLs) also display translocations involving *C-MYC* [8–10], occasionally leading to a diagnostic challenge since aggressive lymphomas with *C-MYC* rearrangement can be either (i) BL, (ii) B cell lymphomas, or unclassifiable, with features intermediate between DLBCL and BL (BCL-U, iBL/DLBCL), or (iii) DLBCL not otherwise specified. The exact classification relies on the interpretation of clinical data, morphology, immunohistochemical profile, and molecular genetics.

In recent years, great efforts have been made to subclassify the heterogeneous group of DLBCL. Gene expression profiling (GEP) stratified DLBCL into biologically and prognostically relevant subtypes based on cell-of-origin (COO) gene signatures, with the activated B cell type being associated with an inferior outcome compared with the germinal center B cell type [11–13]. However, GEP is not yet easily accomplished on formalin-fixed paraffin-embedded (FFPE) tissues as it requires considerably bioinformatics expertise and is available in only a few clinical laboratories, limiting its impact on daily clinical diagnostic use [13]. The translation of complex GEP predictors into immunohistochemical algorithms that assign a COO subtype on the basis of expression of subtype-related proteins has been difficult,

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and the prognostic and predictive accuracies of such algorithms have been shown to be quite variable [14–23]. Recently, c-myc protein overexpression and *C-MYC* gene translocations in DLBCL proved to be a better predictor of prognosis than COO in patients with DLBCL, and not only in the context of double- or triple-hit lymphomas [8, 13, 24–28]. Thus, *C-MYC*/c-myc testing is no longer simply a confirmational analysis for BL, but also provides important information regarding prognosis and risk stratification of DLBCL patients. In light of these emerging findings, practicing hematopathologists are confronted with the following questions:

1. Should any blastic B cell lymphoma be analyzed for *C-MYC* aberrations?
2. When is it appropriate to use immunohistochemistry for c-myc, and when is it more appropriate to perform *C-MYC* FISH?
3. If the above analyses are performed, how should the results be interpreted?

Burkitt's lymphoma

BL is an aggressive, but potentially curable, disease. The translocation involving *C-MYC* is highly characteristic of BL, but not specific; up to 10 % of BL patients may lack demonstrable *C-MYC* translocation by FISH. Neither morphology, genetics, or immunophenotyping can be used as the sole diagnostic criterion for BL, which instead requires a combination of results from different techniques, including clinical presentation (such as age and localization) [29]. This can complicate diagnosis, and the demonstration of a *C-MYC* translocation can assist in attributing a given lymphoma to the corresponding diagnostic entity. Most cases have translocation of *C-MYC* (8q24) to the immunoglobulin heavy chain (14q32) or, less commonly, to the lambda (22q11) or kappa (2p12) light chain loci [29]. Since FISH is a sophisticated technique requiring significant equipment and expertise, immunohistochemistry offers the advantage of being easier to perform and at lower costs. In 2010, Ruzinova et al. [30] described for the first time an antibody detecting c-myc in FFPE that could also serve as a screening tool to identify lymphomas with potential *C-MYC* translocations. However, it is not clear when to start with a "positive result" and when to perform consecutive FISH analysis. The literature provides a range of different cut-offs starting from >50 % positive tumor cell nuclei [31] up to 90–100 % positive tumor cell nuclei [25], which can be explained by the different weighting of the specificity and sensitivity of the respective cut-off scores (Fig. 1). For our biological understanding, the number of positive nuclei should be rather high in BL (since the *C-MYC* translocation is supposed to be the driver mutation, and typically BL contains only a few tumor-infiltrating T cells). Interestingly, most of the known point mutations of *C-MYC* in BL cluster in the N-terminal domain (between p. 1 and 170) [32], a protein region to which the commercially-available c-myc antibody Y69 also binds. Thus, given the frequency of (ongoing) *C-MYC* mutations in BL, it would not be surprising to find immunohistochemically negative BL cases with a detectable *C-MYC* rearrangement by FISH, since a given point mutation may abrogate the binding site of the antibody and, therefore, hide the protein from detection, analogous to "bcl2 negative," yet t(14;18) positive, follicular lymphomas [33].

The recommended algorithm for BL (Table 1) is as follows:

- If morphology, immunohistochemistry ($CD20^+/CD5^-/CD10^+/CyclinD1^-/BCL2^-/BCL6^+/CD44^-/CD38^+/Ki67>95$ %), and clinical context (age and topography) are in line with BL, and c-myc is immunohistochemically detectable in >95 % of tumor cells, diagnosis of BL can be made without FISH confirmation. This phenotype is highly characteristic and allows also the exclusion of blastic mantle cell lymphoma. Importantly, $CD10^-$ and bcl2+BL do not exist.
- All cases expressing c-myc in lower percentages should be subjected to FISH analysis for *C-MYC* (first line break-apart probe and, if negative, double-fusion probe in a second run). If both FISH examinations suggest a non-rearranged *C-MYC* gene, then aggressive "Burkitt's-like" lymphomas (BCL-U) with recurrent 11q aberrations [34] or with c-myc activation due to miRNA-34 downregulation [35] should be considered.
- If everything is in line with BL but c-myc immunohistochemistry is completely negative, *C-MYC* point mutations, which truncate the protein, should be considered, and *C-MYC* FISH should be performed. If *C-MYC* FISH is also negative in such a case, it should be classified as BCL-U according to the suggestion of Salaverria et al. [34].

Diffuse large B cell lymphoma, not otherwise specified

In DLBCL, the situation is more complex. In unselected DLBCL series, rearrangements of the *C-MYC* gene were discovered in approximately 10 % of cases [9, 10, 36–39]. Of these, 20 to 30 % may have an additional break in the *BCL2*- and/or *BCL6* genes [40–42], fulfilling the criteria of so-called genotypic double-hit lymphomas.

Recent data point to the additive prognostic information obtained from FISH for individual risk estimation, since all types of *C-MYC* rearrangements, "IG and non-IG," were

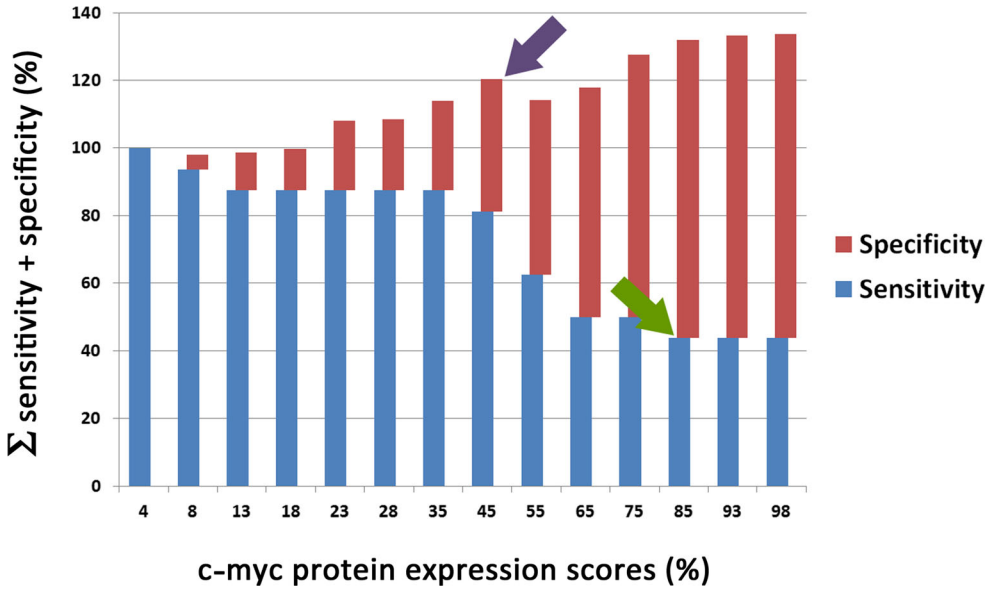
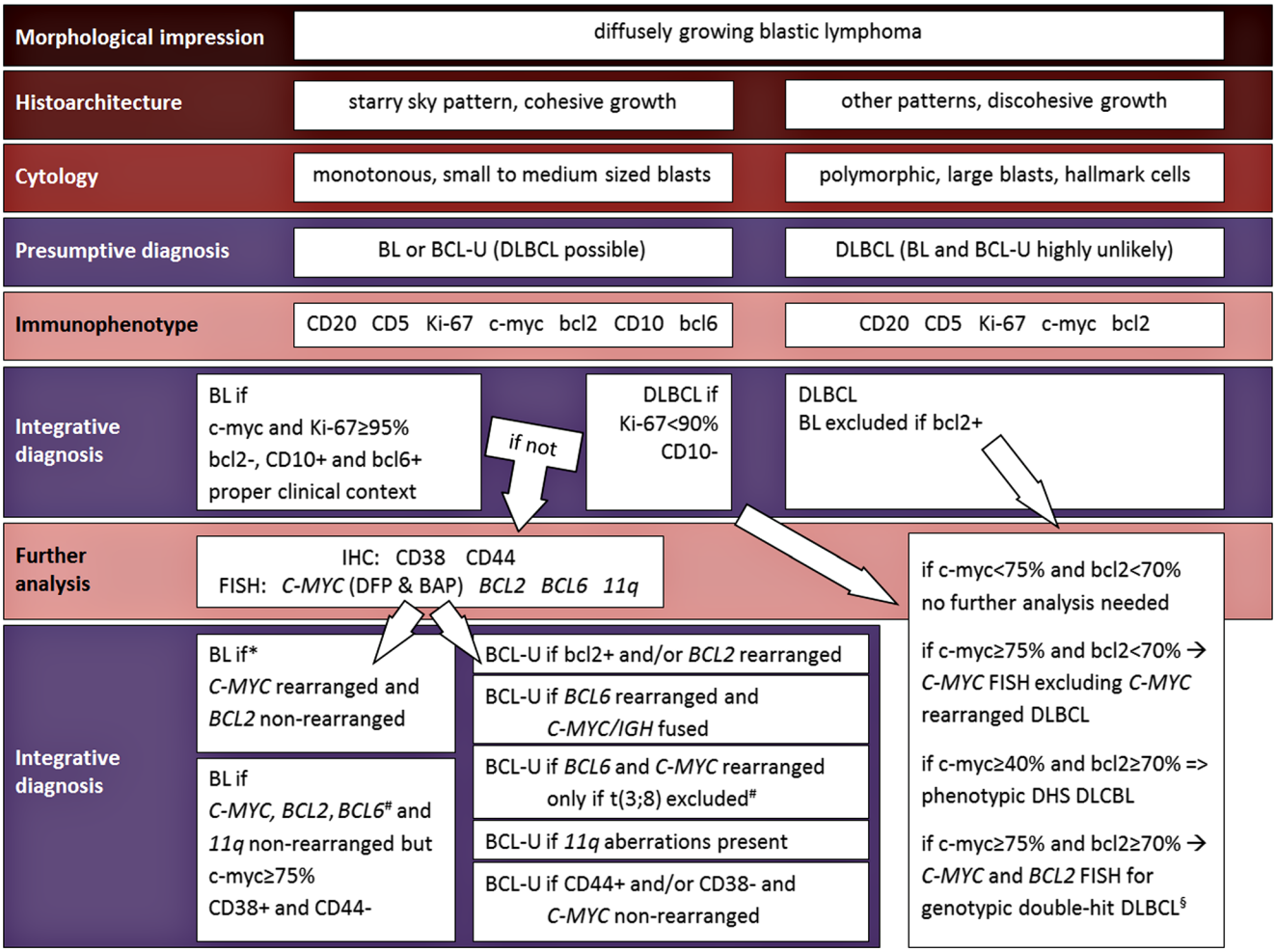


Fig. 1 Specificity and sensitivity of different c-myc cut-off scores to predict *C-MYC* rearrangements. Increasing specificity, but decreasing sensitivity, of different c-myc immunohistochemical cut-off scores to predict *C-MYC* gene rearrangements. Ideally, the sum of specificity and sensitivity would be 200 %. Cut-off scores around 95 % have a specificity and sensitivity sum of approximately 135 % and are nearest to ideal in that they are very specific but less sensitive. Note that to the right of the cut-off score of 85 % (*green arrow*) there is a plateau with respect to sensitivity, indicating that beyond this cut-off, the number of false-negative cases does not increase, but the maximum specificity is reached (maximum amount of true positive cases) at cut-off scores around 95 %. Note that to the right of the cut-off score of 40 % (*purple arrow*) there is a volatile rise in specificity of c-myc immunohistochemistry to predict *C-MYC* rearrangements without significant loss of sensitivity

associated with poorer disease-specific survival in DLBCL [8]. Several studies demonstrated that immunohistochemical staining for c-myc is a good screening tool, since almost 50 % of cases expressing c-myc in over 90 % of tumor cells are also *C-MYC* rearranged. There are also cases (approximately 10 %) that present without (or without relevant) immunohistochemically stainable c-myc despite bearing a rearrangements [26, 27, 43]. The significance of the latter set of cases (*C-MYC* FISH-positive, but immunohistochemically negative) is unclear; additional studies, including identification of the *C-MYC* translocation partners and sequencing for epitope-abrogating point mutations, will be required to further clarify the biological and clinical significance of these subsets [44]. Importantly, recent evidence shows that rearrangements with low c-myc protein expression might be associated with a better prognosis than the presence of rearrangements or high c-myc expression [8], pointing towards the relevance of c-myc protein overexpression. From the technical point of view, it should be stressed that, analogously to BL, it is essential not to use only the break-apart probe or the double-fusion probe in an isolated manner for the detection of *C-MYC* rearrangements, since a subset of translocations may be missed by either probe [8, 45]. The best practical way is to use the double-fusion probe, if in a first attempt the break-apart probe is negative (see below).

The puzzling role of c-myc overexpression in DLBCL can be sought in the fact that, not only the *C-MYC* translocation itself, but particularly the deregulation of c-myc by other mechanisms, can give rise to a BL-like GEP pattern [7]; in the group of lymphomas showing typical gene expression profiles of BL, there were cases without any detectable translocation of *C-MYC* (4 cases of 38). These findings were confirmed by recent studies showing significantly elevated c-myc protein expression in tumors lacking translocations of the *C-MYC* gene [24, 43, 46]. Alternative mechanisms equally presaging upregulation of *C-MYC* have been suggested. miRNAs regulating c-myc expression have been described and, accordingly, miRNA profiles show differences in *C-MYC*-rearranged and non-rearranged BL [35, 47], and also in DLBCL [48]. Amplifications of *C-MYC* (encountered in <1 % of DLBCL) have also been shown to be associated with c-myc overexpression and poorer prognosis [49, 50]. Therefore, given the important role of the c-myc protein, which regulates a substantial number of genes, and possible alternative causes (other than *C-MYC* gene rearrangements) of c-myc overexpression, it is not surprising that protein overexpression of c-myc, regardless of gene translocations, plays an important prognostic role in DLBCL. Moreover, DLBCL with immunohistochemical c-myc overexpression, particularly in combination with bcl2 positivity (referred as phenotypic double-hit score (DHS) positive cases), follows an aggressive clinical course with adverse prognosis [13, 24, 26, 28, 43]. The adverse effect of c-myc deregulation (overexpression on protein level, mRNA level, or

Table 1 The Basel algorithm in diffusely growing blastic lymphomas



The proposed workflow model reflects our approach based on integration of the diagnostic criteria of the WHO 2008 classification and most recent research data, although we acknowledge the scarcity in the literature of data regarding the step describing how to deal with CD10-negative small blastic ("atypical Burkitt-like") DLBCL. Diagnostic cut-off levels of immunohistochemical markers were chosen as follows: ≥7.5 % for CD10 [64] and positive/negative for CD44 and CD38 [65]

*Consider truncating *C-MYC* mutations in c-myc-negative, but FISH-positive, cases

BCL6 breaks in cases positive for *C-MYC/IGH* fusion precludes diagnosis of BL. In instances with *BCL6* and *C-MYC* breaks, t(3;8) juxtaposing *C-MYC* and *BCL6* must be excluded since such cases are still compatible with BL

§ According to the WHO 2008 classification, genotypic double-hit DLBCL can be classified as BCL-U

rearrangement) was recently confirmed by a meta-analysis of 24 eligible studies comprised of 4,662 patients who did or did not have Rituximab as part of their chemotherapy regimen [51].

There are still many open questions before this knowledge can be applied in daily clinical practice:

1. Is it recommended that all DLBCL be stained for c-myc (and bcl2)?
2. What are the best prognostic cut-off values for c-myc (and bcl2)?
3. Which are the most relevant immunohistochemical cut-off values predicting *C-MYC* breaks in DLBCL?
4. When should *C-MYC* FISH be performed?

(a) Considering recent data from a large, independent patient cohort [24, 25, 28, 43], we recommend staining of all DLBCL for both bcl2 and c-myc at initial diagnosis (Table 1) [52]. As is true for all immunohistochemically assessed biomarkers, specific attention should be paid to proper fixation, robust staining protocols, and precise counting. The information gained from these stainings can help physicians to stratify the cohort of DLBCL patients into patients with superior and inferior prognosis even though we acknowledge that a subgroup of high-risk patients such as elderly patients or patients with significant comorbidities cannot be treated more aggressively (see discussion below).

(b) In the literature, different prognostic cut-off scores for c-myc and bcl2 were reported, varying from ≥10 % [26],

≥40 % [24, 28, 43, 44] and >75 % [8] for c-myc, and ≥30 % [44], ≥50 % [26, 43], and ≥70 % [24, 28] for bcl2. The cut-offs most often used (and reproduced) for prognostication are ≥40 % for c-myc and ≥50 or ≥70 % for bcl2.

(c) The question of the most relevant immunohistochemical values for c-myc is difficult to answer, since there are both immunohistochemically negative/FISH positive cases and immunohistochemically positive/FISH negative cases. The optimal immunohistochemical cut-off for c-myc to predict FISH-positive cases is unclear, and data in the literature vary widely between a minimum of ≥30 % [26], ≥50 % [31], ≥80 % [25], and >95 % [8], although lower percentages also exist, e.g., 5 and 19 % [26]. This difference is obviously due to the different weighting of the specificity and sensitivity of the respective cut-off scores (Fig. 1).

In our series of 432 cases, a positive predictive value of c-myc protein overexpression to detect *C-MYC* rearranged cases was 60 % using a cut-off of >95 % [8]. This value was established by analysis of the area under the receiver operating characteristic curves, and had a maximum specificity (36 %) and sensitivity (91 %) to predict *C-MYC* breaks (area=0.599, 95 % confidence interval 0.484–0.715, P= 0.063).

(d) From a practical point of view, we would recommend the following approach (Table 1):

- If c-myc is expressed in ≥40 to <75 % and bcl2 in ≥70 % of tumor cells, it is classified as DLBCL with double hit score 2 (DHS-2).
- If c-myc is expressed in >75 % of tumor cells, FISH is recommended to identify *C-MYC* rearranged cases as well as to identify cases with c-myc upregulations resulting from mechanisms other than translocations. Only if *C-MYC* is rearranged and bcl2 is expressed in >70 % of tumor cells is *BCL2* FISH recommended, since double *BCL2*/*C-MYC* translocated cases (genotypic double-hits) have a very poor prognosis [53]. An additional *BCL6* translocation does not seem to have a significant prognostic impact [8, 27], although this remains controversial [54, 55]. Moreover, *BCL6* is often found to be involved in so-called triple-hit lymphomas together with *BCL2* and *C-MYC* translocations [42].
- If c-myc is expressed in 40 to 74 % of the tumor cells, and *BCL2* in <70 %, FISH can be omitted, since the probability of detecting rearranged *BCL2* is very low, and "*C-MYC*-only" rearranged cases with low c-myc protein expression levels seem to have a prognosis similar to non-rearranged cases [8].

The main goal of this approach is the identification of patients who may benefit from different therapeutic strategies. In the past, patients with *C-MYC* translocation did very poorly [38] with "CHOP-only" therapeutic regimens; while they improved with R-CHOP regimens, the results are still poor compared to *C-MYC* unrearranged patients. Moreover, recent studies have shown that R-CHOP is not an optimal regimen for patients with phenotypic DHS 2 DLBCL, and is particularly inefficient in *C-MYC*/*BCL2* genotypic double-hit DLBCL [56].

In the light of the favorable outcome of the *C-MYC*-driven BL and the impact of *C-MYC* on proliferation, it is appealing to consider the more aggressive therapies used in BL for this group of DLBCL patients. However, there is as yet no published data demonstrating that patients with "double-hit" DLBCL profit from more aggressive therapies [56]. Currently, the US Intergroup is studying a dose-adjusted R-EPOCH protocol (rituximab plus etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin) for *C-MYC*-driven DLBCL, including patients with double-hit biology. This regimen has shown efficacy in BL [57] and in isolated *C-MYC*-rearranged cases, but not in genotypic double-hit DLBCL [55]. More importantly, the median age of these patients is 69 years, meaning that the majority will not be able to tolerate this regimen because of toxicity [55]. Therefore, while extremely promising, much work remains to be done toward the goal of improving the prognosis of these patients.

B cell lymphoma, unclassified

The current WHO classification system defines this category as a lymphoma that has both morphological and genetic features of DLBCL and BL, but cannot be decisively attributed to either disease entity. This includes morphology (cells not typical of BL) as well as immunohistochemistry (lacking the full BL phenotype) and genetics. *C-MYC* FISH is mandatory in this category, since 30–50 % show a *C-MYC* gene translocation. Excluded from this category are typical BL without detectable *C-MYC*-rearrangement, phenotypic double-hit DLBCL, and otherwise clearly defined entities such as blastoid mantle cell lymphomas. However, according to the definition, transformed follicular lymphomas (FL) can also fall into this category [29], which is problematic since transformed FL are genetically and biologically different from aggressive lymphomas arising independently from underlying low-grade lymphomas. In contrast to de novo DLBCL, FL present with *STAT6*, *ARID1A*, and *FAS* mutations and or deletions, as well as aberrations of *MLL2*, *CREBBP*, and *BCL2* [58]. Approximately 35–50 % of these cases have *C-MYC* translocations, especially non-*IG*/*C-MYC* translocations [29].

According to the data of Hummel et al. [7], rare DLBCL (7/114, 6 %) may show a true BL-signature on GEP and may be missed by the current definition based on morphology,

immunohistochemistry, and FISH diagnostic/cytogenetics. Finally, there are cases classified as BL or BCL-U that lack *C-MYC*-translocation but present with chromosome 11q aberrations, characterized by interstitial gains including 11q23.2-q23.3 and telomeric losses of 11q24.1-qter [34], indicating a molecularly distinct subset of B cell lymphomas reminiscent of BL.

BCL-U are considered aggressive lymphomas with a shortened overall survival time [29, 59], and recent data suggests that *C-MYC* is most probably the main cause for this influence on prognosis, while BCL-U cases without *C-MYC* alterations do not differ from DLBCL cases [44]. Similar observations have been made in DLBCL based on expression of bcl2 and c-myc, which abrogate the prognostic significance of the germinal center type/non-germinal center type DLBCL categories based on GEP data [28].

The central role of the *C-MYC* oncogene in the pathogenesis of aggressive lymphomas warrants further clinical research. However, the c-myc protein itself appears to be an undruggable target given that efforts to reduce *C-MYC* expression or interfere with its interaction partner, MAX, have not been successful to date. Novel approaches will target BRD4, a member of the bromodomain and extraterminal superfamily proteins (BET) [60, 61]. BRD4 regulates c-mycexpression directly and indirectly via miRNA expression [62], and interference with BRD4 by small molecules (JQ1 and iBET) has shown significant downregulation of c-myc [63]. Such an interfering small molecule GSK525762 is currently being tested in a phase I study for relapsed hematologic diseases (NCT01943851). If the initial findings regarding *BRD4* and *C-MYC* can be translated into clinical practice, *C-MYC* status could become not only a prognostic, but also a predictive marker for BL, DLBCL, and other *C-MYC*-driven lymphoproliferative diseases, e.g., multiple myeloma and plasmablastic lymphoma.

Conclusion

The risk stratification of the clinically heterogeneous group of DLBCL is still ongoing. Recently, C-MYC and BCL2 have emerged as promising prognostic (immunohistochemical and genetic) markers that clearly stratify DLBCL patients into different risk groups. Unresolved issues include the fact that double-hit DLBCL (on the genetic level) and DHS 2 DLBCL (on the protein expression level) are not the same despite significant overlap between these two groups, and it remains to be determined whether risk stratification by FISH is superior to immunohistochemistry or vice versa. From our point of view, immunohistochemistry has advantages such as practicability, short turn-around time. and low cost, but FISH analysis remains an indispensable tool in some of the above-described situations and cannot be completely replaced by immunohistochemistry. Considering that immunohistochemical stainings with the anti-c-myc antibody is a very recent methodology with limited experience, we recommend the use of a combined FISH and immunohistochemical model (Table 1). In light of the upcoming therapeutic options such as small molecules that downregulate *C-MYC*, pathologies should be ready for adequate determination of *C-MYC* status.

Conflict of interest The authors declare that they have no conflict of interest.

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