MYC protein expression scoring and its impact on the prognosis of aggressive B-cell lymphoma patients

This study examined the reproducibility of MYC and BCL-2 immunohistochemical scoring as well as the impact of higher expression of both proteins (double expressor status, DE) on survival and progression in a large retrospective cohort of aggressive B-cell lymphoma patients treated with rituximab *plus* cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) or R-CHOP-like regimens with a median follow up of 67 months (range 0-138). We also investigated possible MYC protein expression cut offs with the highest reproducibility among pathologists and predictability of gene translocation. We showed that immunohistochemistry (IHC) for MYC and BCL-2 is highly reproducible when cut-off values of >70% for MYC and >50% for BCL-2 are used. This threshold not only predicts the presence of rearrangements (with respect to *MYC*), but is also clinically valuable. In fact, it identifies a subset of patients who are poor responders and who may benefit from alternate therapeutic strategies.

Several investigators have demonstrated that diffuse large B-cell lymphomas (DLBCL) with MYC and BCL-2 double expression (DE-LBCL) have adverse prognosis when treated by conventional immunochemotherapies.[1] Therefore, evaluation of MYC and BCL-2 protein expression by IHC is an important tool in the prognostic stratification of patients.[2] Although expression of MYC-IHC in ≥40% neoplastic cells and the BCL-2 in ≥50% have been indicated as prognostically significant cut offs in many reports, among hematopathologists, some disagreement about these thresholds remains.[3,4] Standardizing cut offs to define positivity for MYC-IHC and BCL-2-IHC with higher reproducibility and prognostic impact is thus highly desirable in order to optimize patient management.[5,6]

We analyzed 753 aggressive B-cell lymphoma patients. These included: Burkitt lymphomas (BL, n=223); DLBCL not otherwise specified (DLBCL-NOS, n=456); high grade B-cell lymphomas (HGBCL) with *MYC* and *BCL-2* and/or *BCL-6* rearrangement [HG double hit (HGDH), n=51, including 8 blastoid, 16 diffuse monomorphic and 27 intermediate morphology]; and HGBCL, NOS (n=23).[7-9] All cases belonging to these categories, except for BL, will be referred to hereon as non-BL. The following clinical data were collected for non-BL: sex, age at diagnosis, ECOG performance status, stage (Ann Arbor), lactate dehydrogenase (LDH) level, International Prognostic Index (IPI), bulky disease, extranodal *versus* nodal, bone marrow involvement. Original MYC-IHC and BCL-2-IHC slides were re-evaluated by 3 trained pathologists blinded as to the original scores. Although aware that immunohistochemical interpretation may be affected by technical issues, we intentionally decided not to re-stain all slides in only one laboratory, but instead to confront original slides from different laboratories which apply different unmasking procedures and use different clones on a "real-life" basis (Table 1). Scores were reported in 5% intervals rounded up to the nearest 10%. Any discrepancies in MYC-IHC and BCL-2-IHC scoring was resolved by consensus on a multi-head microscope until an agreement of >95% concordance was reached. Discrepancy was defined as 10% deviation. Our findings confirm that BCL-2-IHC scoring is highly reproducible across the different institutions and that a ≥50% cut off is reliably assessable [agreement >90%; k=0.97; standard error (SE):=0.018; 95% confidence interval (CI): 0.765-0.901]. On the contrary, scoring MYC-IHC staining was confirmed to be critical, mainly because of the variability of staining intensity, percentage of positivity, presence of necrosis and crush artifacts. Concordance was high only for MYC-IHC positivity >70%, while a larger discrepancy was observed in the range 40-69%[10] (Figure 1). The latter can have a crucial impact on clinical decision making

Table 1. Immunohistochemical and FISH analysis of MYC* and BCL-2* proteins and genes in the different institutions.

	MYC clone/source	MYC antigen retrieval/dilution	BCL-2 clone/source	BCL-2 antigen retrieval/dilution
Berlin	EP121, Epitomics, Germany	Pressure cooker, citrate buffer pH 6.0	124, Agilent, Germany	Pressure cooker, citrate buffer pH 6.0
Bologna	EP121, Epitomics, Milan	PT link 92°C /1:100	124, Agilent, Milan	PT link 90°C /1:100
Firenze	Y69, Roche, Milan	Ready to use	SP124, Roche, Milan	Ready to use
London	Y69, Abcam, UK	Ready to use	124, Dako, UK	PT link 90°C /1:100
Siena & Nairobi	Y69, Roche, Milan	Ready to use	SP124, Roche, Milan	Ready to use
	MYC BAP probe/source	MYC FUSION probe/source	BCL-2 BAP probe/source	
Berlin	Split Signal MYC, Agilent, Germany	Dual fusion IGH/MYC, Zytomed, Germany	Splits Signal BCL-2, Agilent, Germany	
Bologna	ZytoLight SPEC MYC Dual Color Break Apart Probe, Bio-Optica, ZitoVision GmbH, Germany	ZytoLight SPEC MYC/IgH Dual Color Dual Fusion Probe , Bio-Optica, ZitoVision GmbH, Germany	ZytoLight SPEC BCL-2 Dual Color Break Apart Probe, Bioptica, ZitoVision GmbH, Germany	
Firenze	ZytoLight SPEC MYC Dual Color Break Apart Probe, Bio-Optica, ZitoVision GmbH, Germany	ZytoLight SPEC MYC/IgH Dual Color Dual Fusion Probe, Bio-Optica, ZitoVision GmbH, Germany	ZytoLight SPEC BCL-2 Dual Color Break Apart Probe, Bioptica, ZitoVision GmbH, Germany	
London	Kreatech, Leica, UK	Kreatech, Leica, UK	Kreatech, Leica, UK	

Siena & NairobiZytoLight SPEC MYC Dual Color Break Apart Probe, Bio-Optica, ZitoVision GmbH, Germany ZytoLight SPEC MYC/IgH Dual Color Dual Fusion Probe, Bio-Optica, ZitoVision GmbH, GermanyZytoLight SPEC BCL-2 Dual Color Break Apart Probe, Bioptica, ZitoVision GmbH, Germany. *On 4 m thick formalin-fixed paraffin-embedded sections; BAP: break-apart probe.

Concordance of MYC IHC scoring: original versus revised
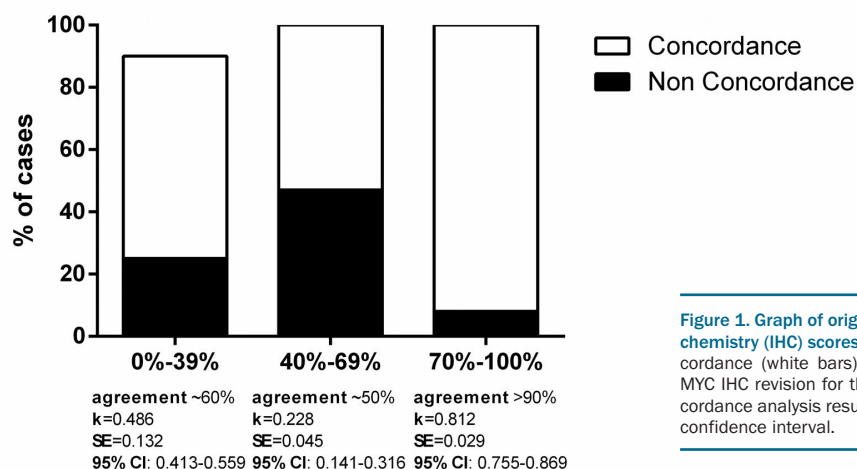

Figure 1. Graph of original and revised MYC immunohistochemistry (IHC) scores. The percentage of cases with concordance (white bars) or discordance (black bars) after MYC IHC revision for the three ranges is shown. The concordance analysis results are listed. SE: standard error; CI: confidence interval.

and patient care, as well as on the comparison between different studies. Therefore, we propose the threshold of MYC-IHC 70-100% to define non-BL cases with high expression of MYC and 0-39% to classify samples with low expression of MYC protein. Samples with MYC-IHC scores between 40% and 69% could not be categorized as MYC protein negative or positive with acceptable accuracy in routine practice and should be termed as cases with intermediate MYC expression. Moreover, we suggest scoring should preferably be defined on moderately to strongly stained nuclei and in hot spot areas, if present. After revision, in the non-BL series, prevalence of DE-LBCL as defined by the cut offs proposed by the literature (MYC-IHC ≥40% and BCL-2-IHC ≥50%) was 32% (n=176/530);[7] of these 100 showed MYC-IHC ≥70% (DE 70% in the following).

A second end point of this study was to investigate the relation between MYC-IHC and *MYC* gene alterations, trying to identify parameters potentially applicable in a value-for-money approach. When using the threshold of MYC-IHC ≥80% in BL and ≥70% in non-BL, around 99% BL and 88% non-BL cases with *MYC* rearrangements can be predicted. Therefore, considering the high correlation between *MYC* gene rearrangement and MYC protein expression in BL, FISH analysis could theoretically be limited to cases with atypical morphology and/or immunophenotype and MYC-IHC <80%. In non-BL, a cut off of MYC-IHC ≥70% would identify cases at higher probability of MYC rearrangement. However, it should be noted that roughly 10% of HGDH have a lower expression of MYC; in these cases, FISH should be recommended.[11]

The third end point was to perform a survival analysis in cases of HGDH, DE, nonDH/nonDE patients for whom overall survival (OS) and progression-free survival (PFS) data were available. HGDH lymphomas are known to be very aggressive neoplasms that do not respond to standard therapy and they have a dismal prognosis.[12] However, it has recently been reported that the adverse prognosis may be mitigated by other factors such as lack of BCL-2 protein, and absence/low MYC protein expression.[5] The clinical significance of MYC-IHC and BCL-2-IHC expression has been extensively studied but the results have not been consistent; hence, there is still no clear use for protein expression evaluation in the clinic.[13,14]

Univariate Cox analysis showed that the factors significantly associated with OS and PFS were: age [Odds Ratio (OR): 4.8% each year; confidence interval (CI): 1.03-1.066; *P*<0.001], LDH >150 UI/L (OR: 187%, CI: 1.756-4.715; *P*<0.001), IPI (OR: 71%, CI: 1.422-2.056; *P*<0.001), bone marrow involvement (OR: 86.2%, CI: 1.102-3.145; *P*=0.02), *MYC* translocation (OR: 71%, CI: 1.281-2.297; *P*<0.001), *BCL-2* translocation (OR: 97%, CI: 1.389-2.795; *P*=0.002), MYC-IHC ≥70% (OR: 65%, CI: 0.22-0.539; *P*<0.001), DH status (OR: 310%, CI: 2.529-6.662; *P*<0.001), DE 70% status (OR: 61.5%, CI: 1.976-2.67; *P*=0.04). Stepwise multivariate analysis demonstrated that IPI (OR: 62.2%, CI: 1.225-2.146; *P*=0.001), MYC-IHC ≥70% (OR: 224.8%, CI: 1.714-6.155; *P*<0.001), DH status (OR: 131.7%, CI: 1.003-5.350; *P*=0.049), DE 70% status (OR: 177.5%, CI: 1.020-1.080; *P*=0.006) were independent prognostic factors that influence patients' outcome.

A comparison of HGDH, DE-LBCL [according to World Health Organization (WHO) criteria, i.e. MYC-IHC ≥40% and BCL-2-IHC ≥50%] and nonDH/nonDE subsets of non-BL in terms of OS and PFS confirmed that HGDH patients showed significantly worse survival than the other two groups (*P*<0.001; χ^2 39.24 for OS and 13.13 for PFS) (Figure 2A and *Online Supplementary Figure S1A*).

By stratifying DE-LBCL according to MYC protein expression (70-100%, 40-69%,) the prognosis turned out to be poorer in the former (*P*<0.001, $\chi^2$13.152 for OS and 10.723 for PFS) (Figure 2B and *Online Supplementary Figure S1B*).

Dividing HGDH cases according to MYC protein positivity revealed that those with MYC-IHC ≥70% had a worse prognosis than cases with MYC-IHC ≤69% (*P*<0.001, $\chi^2$48.215 for OS and 35.549 for PFS) (Figure 2C and *Online Supplementary Figure S1C*). This supports prior observations that patients with *MYC* rearrangements or DH lymphomas devoid of MYC-IHC expression might exhibit less aggressive clinical behaviors.[13]

Given that the highest concordance and reproducibility of MYC-IHC was >70%, we compared OS and PFS of DE-LBCL showing MYC-IHC >70% and BCL-2-IHC >50% with HGDH cases; we found no significant differ-
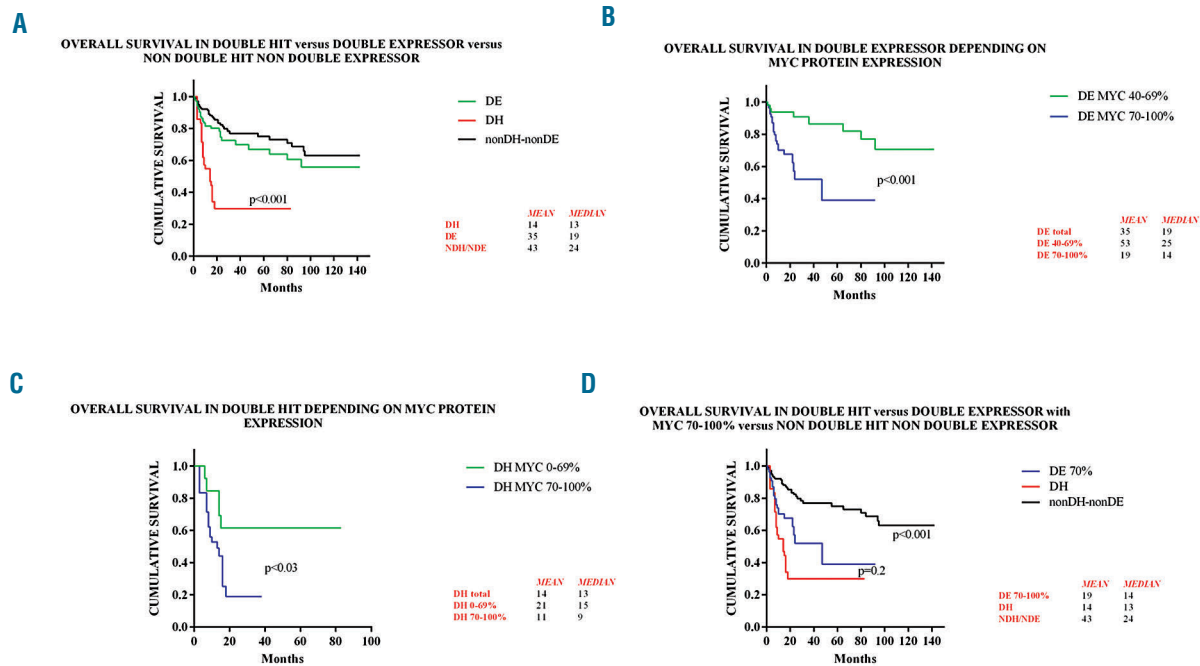
Figure 2. Kaplan-Meier curves of overall survival (OS) in the different patient groups. (A) Kaplan-Meier curve of OS in double hit (DH) *versus* double expressor (DE) *versus* non-double hit/non-double expressor (nonDH/nonDE) lymphomas. There was a significant difference in OS between the DH and the other two groups (log rank test, *P*<0.001). DH lymphomas were defined by having both *MYC* and *BCL-2* rearrangements; DE were characterized by showing MYC-immuno-histochemistry (IHC) ≥40% and BCL-2-IHC ≥50%; nonDH/nonDE included cases without double hits or with one only single hit, and with none or only one protein expression between MYC and BCL-2. (B) Kaplan-Meier curve of OS in DE depending on MYC protein expression. OS was significantly lower in the group showing MYC-IHC ≥70% than in the group with MYC-IHC 40-69% (log rank test, *P*<0.001) indicating that a MYC-IHC ≥70% cut off defines a clinically relevant subgroup of DE cases. (C) Kaplan-Meier curve of OS in DH depending on MYC protein expression. There was a significant difference in OS between the two groups (MYC-IHC 0-69% and BCL-2-IHC ≥50%, and MYC-IHC ≥70% and BCL-2-IHC ≥50%) with MYC ≥70% cases having the lower survival rate (log rank test, *P*<0.001). Noteworthy, among HGDH with MYC-IHC 0-69%, the 3 cases showing MYC-IHC <40% had a mean OS of 44 months (*data not shown*). These findings indicate that adverse survival in HGDH is highly impacted by high MYC-IHC expression (≥70%). (D) Kaplan-Meier curve of OS in DH *versus* DE as defined by MYC-IHC ≥70% and BCL-2-IHC ≥50% *versus* nonDH/nonDE. OS overlaps in DH and DE with MYC-IHC ≥70% and BCL-2-IHC ≥50% (log rank test, *P*=0.2 for DH *versus* DE with MYC ≥70% and BCL-2-IHC ≥50%;), this is worse than the OS of the nonDH/nonDE (log rank test, *P*<0.001 for either DH or DE with MYC ≥70% and BCL-2-IHC ≥50% *versus* nonDH/nonDE).

ences in prognosis (*P*=0.2, $\chi^2$0.777 for OS and 3.441 for PFS) (Figure 2D and *Online Supplementary Figure S1D*).

Interestingly, the OS and the PFS of HGBCL, NOS strictly depended on *MYC* expression being 30 and 28 months in HGBCL, NOS with MYC 40-69%, 10 and 14 months in those with MYC 70-100%.

These findings again highlight the need to establish more widely reproducible cut-off values for IHC assays with higher predictive value, to safely stratify high-risk patients. Moreover, they may also, at least in part, explain the above mentioned inconsistencies in published data.

Finally, since the cell-of-origin (COO) classification of LBCL exerts a prognostic impact, with non-GCB lymphomas showing a poorer prognosis than GCB ones, we stratified HGDH and DE-LBCL according to COO defined by Hans' algorithm.[15] A poorer prognosis was demonstrated only in patients with MYC-IHC ≥70%, with no differences according to the COO. However, no conclusions can be drawn due to both the limited number of cases examined per group and to the IHC assessment of COO which is known to misclassify a subset of samples.

Different molecular mechanisms may be responsible for the same disease entity and neoplastic phenotype. FISH currently represents the gold standard for identifying rearrangements, but it is not capable of detecting genetic deregulation affecting gene expression at tran-scriptional and post-transcriptional levels that might result in protein overexpression and neoplastic transformation.[4] Our results support the role of MYC protein as the active trigger of the MYC-mediated oncogenic effects since protein expression levels likely represent a more direct measure of the activity of a particular gene[16] and MYC-IHC should be undertaken in all cases. However, the last WHO criteria assessed that MYC staining is not reliable enough to select cases for FISH analysis.[7] According to our results, the cut off of MYC-IHC ≥70%, based both on reproducibility among pathologists and clinical impact, is a good indicator of underlying *MYC* gene rearrangement. Nonetheless, our promising data need further validation in an independent cohort of patients. In addition, given the highest scoring efficiency, the cut offs of MYC-IHC ≥70% and BCL-2-IHC ≥50% could be applied when FISH is not available (due to issues of practicability, cost, short turn-around-time, quality control) to select a subset of patients who are poor responders and who may benefit from alternate therapeutic strategies.

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