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Diffuse large B-cell lymphoma cell-of-origin classification using the Lymph2Cx assay in the context of BCL2 and MYC expression status

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Potential Conflicts of Interests

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The discovery of two distinct gene expression profiles (GEP) with either a germinal center B-cell (GCB) or activated B-cell (ABC) cell-of-origin (COO) within diffuse large B-cell lymphoma (DLBCL) advanced the understanding of the heterogeneous nature of DLBCL [1]. Follow-up GEP and sequencing studies confirmed the molecular and prognostic distinction of GCB-DLBCL and ABC-DLBCL. Recognizing the need for accurately and efficiently identifying COO in commonly prepared formalin-fixed paraffin-embedded tissues (FFPET) with a robust assay that utilizes a small probe set and rapid output, we established the Lymph2Cx assay, which uses 20 gene probes and Nanostring technology (Seattle, WA) to classify DLBCL into reproducible COO subgroups [2]. Expression of the anti-apoptotic BCL2 and proliferative MYC oncogenes are also highly associated with prognosis, such that patients with tumors overexpressing both proteins (dual expressers) and those that harbor concurrent *BCL2/MYC* translocations (double-hit) have a worse outcome [3–6]. While many studies have shown the prognostic importance of both COO and BCL2/MYC, in one study BCL2/MYC expression was reported to over-ride the prognostic significance of COO [7]. The availability of targeted therapies towards both ABC-DLBCL associated aberrant pathways and BCL2 for clinical trials, with the potential for others in the near future including anti-MYC compounds, places considerable importance on identifying patients at high-risk for aggressive, refractory disease [8–11]. As such, our current objective was to further investigate the relationship between COO, as determined by Lymph2Cx, with BCL2 and MYC protein status.

We evaluated BCL2 and MYC expression in 56 DLBCL cases that included 20 ABC, 27 GCB, and 9 unclassified from a subset of the Scott [2] et al series used to establish the Lymph2Cx assay. It is important to note that the 56 cases were also from the Lenz [12] et al series. These highly annotated cases had sufficient FFPET material remaining to core for tissue microarray (TMA) construction. Immunohistochemical (IHC) analyses were performed on the TMA using the standard anti-BCL2 clone 124 and the new rabbit monoclonal anti-BCL2 clone SP66, which is reportedly more sensitive for IHC, and the anti-MYC clone Y69 [13] (Figure 1A). The stained TMAs were independently scored in increments of 10% by two hematopathologists (K.T. and L.M.R.) and BCL2 (50%) and MYC (40%) positivity were set at cut-offs of as previously described [3]. Discrepancies in scoring were resolved by a third observer. There was a 93% scoring concordance between the two pathologists for assessment of MYC staining, 94% for BCL2 with clone 124, and 100% for BCL2 with clone SP66. Approval for this study was obtained from the University of Arizona Institutional Review Board in accordance with the Declaration of Helsinki.

Overall, ABC-DLBCL (n=20) displayed worse disease specific (DSS) and progression free survival (PFS) compared to GCB-DLBCL (n=27) using the Lymph2Cx assigned COO from our previous study [2] with PFS reaching significance (log rank $P=0.01$; Supplemental Figure 1A, i). The survival endpoints were defined according to the International Working Group response criteria [14] and estimated using the Kaplan-Meier method. These correlations were slightly more pronounced compared to analysis with COO determined from the frozen tissue Affymetrix GEP [12], which achieved a PFS difference with $P=0.08$ (Supplemental Figure 1A, ii). This discrepancy in prognostic significance is due to the ability of the Lymph2Cx assay to resolve more of the unclassifiable cases into a ABC or GCB subgroup. Also consistent with the literature, BCL2 protein expression was detected

more frequently in ABC than GCB either alone (70% vs 55%) or concurrently with MYC (50% vs 30%), using SP66 and to a lesser extent clone 124 (BCL2 alone: 55% vs 44% and BCL2/MYC: 35% vs 26%). The SP66 antibody was superior for BCL2 detection and similar in demonstrating the prognostic significance of BCL2+/MYC+ cases in the total cohort compared to the 124 clone. Most likely, the discrepant staining was due to the potential for 124 false-negatives as previously described [13]. Consistent with this, the SP66 antibody BCL2 positivity rate was higher (70%, 39/56 cases vs 55%, 31/56 cases).

The Lymph2Cx COO designations also reflected the known correlation of *BCL2*t(14;18) translocations predominantly with GCB-DLBCL (38%, 9/24 cases vs 0% 0/18 ABC cases, $P=0.005$) within the cohort, which displayed the typical overall frequency (19%, 9/47 cases). We confirmed that BCL2, when co-expressed with high levels of MYC protein, portends a poor clinical outcome in the whole DLBCL case series using either clone 124 ($n=14$; Supplemental Figure 1B, i) or SP66 ($n=19$; Supplemental Figure 1B, ii) to detect BCL2 expression. We further demonstrate the BCL2+/MYC+ phenotype identified these patients within both GCB ($n=7$; Figure 1B, i and iii) and ABC subgroups ($n=7$; Figure 1B, ii and iv). When BCL2+/MYC+ cases were examined according to subtype, ABC-DLBCL displayed a worse DSS and PFS (Figure 1C, i and iii), although there were insufficient cases to reach statistical significance. In all other cases ($n=33$), BCL2 and/or MYC protein negative, there was no significant difference in DSS between the COO subtypes (GCB: $n=20$; ABC: $n=13$; Figure 1C, ii). However, the ABC COO designation still distinguished a subset of patients with worse PFS (Figure 1C, iv). Within these ABC non-dual expressers, there were 5 BCL2+ cases, 7 MYC+ cases, and 1 case that was negative for both proteins. The corresponding overall survival curves for Figure 1B and 1C are provided in Supplemental Figure 2. We then further stratified the “other” category and compared the survival of patients negative for BCL2/MYC, BCL2+ only (BCL2+/MYC-), and MYC+ only (BCL2-/MYC+) with the dual expressers. In the GCB-DLBCL, there was no significant difference (DSS $P=0.28$ and PFS $P=0.28$) between the groups (BCL2-/MYC-, $n=8$; BCL2-/MYC+, $n=7$; BCL2+/MYC-, $n=5$; BCL2+/MYC+, $n=7$; Figure 1D, i and iii). For the ABC subtype, a worse DSS ($P=0.04$) and a trend towards worse PFS ($P=0.06$) for the BCL2+/MYC+ cases ($n=7$) compared to the BCL2-/MYC+ ($n=7$) and BCL2+/MYC- ($n=4$) was observed; however, there are two patients with ABC-DLBCL tumors negative for both oncoproteins that progressed and succumbed to the disease within one year of initial treatment suggesting that within the ABC classification there are other factors important that contribute to the aggressive nature of this subgroup (Figure 1D, ii and iv). In both the ABC and GCB subtypes BCL2 or MYC expression alone did not confer a worse outcome compared to BCL2+/MYC+ cases. When each COO subset was analyzed for MYC protein expression alone, there was no correlation with patient outcome (ABC, DSS $P=0.63$ and PFS $P=0.88$; GCB, DSS $P=0.99$ and PFS $P=0.81$; data not shown). Similarly, BCL2 protein positivity alone did not significantly correlate with patient outcome within either of the DLBCL subtypes with clone 124 (ABC, DSS $P=0.26$ and PFS $P=0.69$; GCB, DSS $P=0.47$ and PFS $P=0.66$) or SP66 (ABC, DSS $P=0.74$ and PFS $P=0.33$; GCB, DSS $P=0.71$ and PFS $P=0.29$; data not shown). However, the ABC classification tends to identify those patients with worse PFS in BCL2+ only ($P=0.18$ with 124; $P=0.06$ with SP66) or MYC+ only ($P=0.08$) cases (Figure 1E and Supplemental Figure 1C for SP66).

The Lymph2Cx assay provides the most accurate assessment of DLBCL COO and maintains the well-known subtype associations with BCL2/MYC protein and *BCL2* translocation. As such, although relatively small, the selected cohort of highly annotated and genetically profiled cases for this current study are qualified to address the question of whether the ABC subgroup continues to characterize a distinct subset of patients with poor prognosis within aggressive BCL2+/MYC+ DLBCL. Our analyses suggest COO remains prognostically relevant even in the context of BCL2 and MYC protein status; however, there were not enough cases for a multivariate comparison. The ABC-DLBCL subclass consistently tends to associate with poor outcome regardless of BCL2/MYC status. The different methodologies used here, including the cut-off of 50% for BCL2 positivity and the Lymph2Cx assay for COO classification, which allows for high level of accuracy and reliability as well as resolution of more of the unclassifiable cases, compared to those in the Hu et al paper may account for the difference in observing the outcome of the ABC subgroup. Additionally, concurrent BCL2/MYC expression also appears to identify a subset of patients with poor prognosis in both the GCB and ABC subtypes. Further studies using a larger cohort of DLBCL patients would help to clarify these findings and provide insight into recommendations for antibody choice in the clinic to accurately and reliably assess patient prognosis. The ability and need to stratify patients for both COO and oncogene status is important as targeted therapies and precision medicine are on the horizon.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Prognostic significance of DLBCL cell-of-origin and BCL2/MYC protein positivity
 (A) Immunohistochemical analysis of MYC (i), BCL2 with clone 124 (ii), and BCL2 with clone SP66 (iii) in a MYC+ DLBCL case that was BCL2- with 124 but BCL2+ with SP66. Inset in (ii) depicts a representative case that stained BCL2+ with 124. Images were captured at 400x magnification using an Olymups BX-61 microscope system and a Nikon DS-Fil digital CCD camera and DS-L2 imaging controller. (B) Disease free (DSS) and progression free survival (PFS) of DLBCL subtypes as assigned by Lymph2Cx from a previous study [12], GCB (i and iii) and ABC (ii and iv), according to BCL2 and MYC dual expresser protein status. (C) DSS and PFS of concurrent BCL2/MYC protein positive cases (i and iii) in comparison to all other cases (ii and iv) according to DLBCL subtypes. (D) DSS and PFS

of GCB (i and iii) and ABC (ii and iv) according to stratification of BCL2 and MYC protein status. (E) DSS and PFS of BCL2+/MYC- (i and iii) and BCL2-/MYC+ (ii and iv) cases according to ABC and GCB subtypes. All patients received R-CHOP therapy and DSS and PFS were estimated with the Kaplan-Meier method from the date of initial treatment. Analyses with BCL2 detection using clone 124 are shown.