A bioclinical prognostic model using MYC and BCL2 predicts outcome in relapsed/refractory diffuse large B-cell lymphoma

Mark Bosch,[1] Ariz Akhter,[2] Bingshu E. Chen,[3] Adnan Mansoor,[2] David Lebrun,[4] David Good,[3] Michael Crump,[5] Lois Shepherd,[3] David W. Scott[6] and Douglas A. Stewart[2]

[1]University of Saskatchewan, SK; [2]University of Calgary, AB; [3]Canadian Cancer Trials Group, Queen's University, Kingston, ON; [4]Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON; [5]University of Toronto, ON and [6]BC Cancer Agency, Vancouver, BC, Canada

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Correspondence:
douglas.stewart@albertahealthservices.ca

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ABSTRACT

The objective of this study was to create a bioclinical model, based on clinical and molecular predictors of event-free and overall survival for relapsed/refractory diffuse large B-cell lymphoma patients treated on the Canadian Cancer Trials Group (CCTG) LY12 prospective study. In 91 cases, sufficient histologic material was available to create tissue microarrays and perform immunohistochemistry staining for CD10, BCL6, MUM1/IRF4, FOXP1, LMO2, BCL2, MYC, P53 and phosphoSTAT3 (pySTAT3) expression. Sixty-seven cases had material sufficient for fluorescent *in situ* hybridization (FISH) for *MYC* and *BCL2*. In addition, 97 formalin-fixed, paraffin-embedded tissue samples underwent digital gene expression profiling (GEP) to evaluate *BCL2, MYC, P53*, and *STAT3* expression, and to determine cell-of-origin (COO) using the Lymph2Cx assay. No method of determining COO predicted event-free survival (EFS) or overall survival (OS). Factors independently associated with survival outcomes in multivariate analysis included primary refractory disease, elevated serum lactate dehydrogenase (LDH) at relapse, and MYC or BCL2 protein or gene expression. A bioclinical score using these four factors predicted outcome with 3-year EFS for cases with 0-1 *vs.* 2-4 factors of 55% *vs.* 16% ($P<0.0001$), respectively, assessing MYC and BCL2 by immunohistochemistry, 46% vs. 5% ($P<0.0001$) assessing *MYC* and *BCL2* messenger ribonucleic acid (mRNA) by digital gene expression, and 42% *vs.* 21% ($P=0.079$) assessing *MYC* and *BCL2* by FISH. This proposed bioclinical model should be further studied and validated in other datasets, but may discriminate relapsed/refractory diffuse large B-cell lymphoma (DLBCL) patients who could benefit from conventional salvage therapy from others who require novel approaches. The LY12 study; *clinicaltrials.gov Identifier: 00078949.*

Introduction

DLBCL has considerable biologic and clinical heterogeneity.[1] Following standard chemoimmunotherapy, 10-65% of patients will relapse depending upon their presenting International Prognostic Index (IPI) score.[2] Salvage chemotherapy +/- rituximab followed by high dose chemotherapy/autologous stem-cell transplantation (HDCT/ASCT) is the standard treatment for relapsed/refractory DLBCL (rrDLBCL),[3-6] although fewer than half of patients are cured with this approach.[6-9] To date, research assessing predictive and prognostic biomarkers for rrDLBCL has been limited,[10] but with the dawn of multiple novel agents, such research will be critical to help stratify patients into personalized treatment strategies.

The two molecular COO subtypes of DLBCL recognized by the 2016 revised WHO classification are the germinal center B-cell (GCB) and activated B-cell (ABC). Hans,[11] Choi,[12] and the tally[13] algorithms use immunohistochemistry (IHC) to assign COO, however, the clinical significance of COO subtyping using IHC remains controversial, especially for relapsed disease.[14-18] Current data suggest that COO is more strongly associated with prognosis of DLBCL if assessed by GEP rather than by IHC.[19-21] In addition, it is known that genetic aberrations or protein

expression of BCL2 and MYC identified by FISH or IHC, respectively, are associated with a poor prognosis in newly diagnosed DLBCL.[22-28] The objective of this study was to create a bioclinical model, comprising clinical and molecular features, for EFS and OS of rrDLBCL patients utilizing materials and data from the prospective LY12 study conducted by the CCTG.

Methods

In the study herein, we evaluated a subset of the 619 patients enrolled on the LY12 trial that compared the efficacy of GDP(+/- R) (gemcitabine, dexamethasone and cisplatin +/- rituximab) to DHAP(+/- R) (dexamethasone, high-dose cytarabine, and cisplatin +/- rituximab) followed by HDCT/ASCT for patients with relapsed/refractory aggressive histology lymphoma.[3] All patients gave written informed consent to participate and to provide tissue material for biologic studies. This correlative science study was approved by the Health Research Ethics Board of Alberta.

Morphology, IHC, Digital GEP, FISH

Among the 619 patients in LY12, 130 had rrDLBCL and sufficient formalin-fixed paraffin-embedded (FFPE) tissue samples available to create tissue microarrays (TMAs). TMAs were constructed, using triplicate, 1.0 mm cores from each donor paraffin

Table 1. Characteristics of B-cell lymphoma patients.

	All LY12	IHC	NanoString GEP	FISH
Total Number Patients	554	91	97	67
Treatment Arm:		*P*=0.09	*P*=0.06	*P*=0.15
DHAP(+/- R)	277 (50.0)	53 (58.2)	57 (58.8)	39 (58.2)
GDP(+/- R)	277 (50.0)	38 (41.8)	40 (41.2)	28 (41.8)
Sex		*P*=0.55	*P*=0.24	*P*=0.62
Male	332 (59.9)	52 (57.1)	53 (54.6)	42 (62.7)
Female	222 (40.1)	39 (42.9)	44 (45.4)	25 (62.7)
Age, years		*P*=0.69	*P*=0.65	*P*=0.66
Median	55	54.7	55.3	55.4
Range	19-74	28-66	28-66	29-66
ECOG		*P*=0.91	*P*=0.89	*P*=0.58
0-1	483 (87.2)	79 (86.8)	85 (87.6)	57 (85.1)
2-3	71 (12.8)	12 (13.2)	12 (12.4)	10 (14.9)
Relapse Stage		*P*=0.56	*P*=0.94	*P*=0.46
I-II	179 (32.3)	27 (29.7)	31 (32.0)	19 (28.4)
III-IV	375 (67.7)	64 (70.3)	66 (68.0)	48 (71.6)
Serum LDH		*P*=0.95	*P*=0.99	*P*=0.78
Elevated	316 (57.0)	50 (54.9)	56 (57.7)	38 (56.7)
"B" symptoms		*P*=0.05	*P*=0.09	*P*=0.08
Present	185 (33.4)	39 (42.9)	40 (41.2)	29 (43.3)
Extranodal sites		*P*=0.51	*P*=0.60	*P*=0.50
>1	143 (25.8)	21 (23.1)	23 (23.7)	15 (22.4)
Bone marrow		*P*=0.53	*P*=0.42	*P*=0.17
Involved	39 (7.0)	5 (5.5)	5 (5.2)	2 (3.0)
Relapse aaIPI		*P*=0.78	*P*=0.58	*P*=0.26
0-1	208 (37.5)	33 (36.3)	34 (35.1)	21 (31.3)
2-3	346 (62.5)	58 (63.7)	63 (64.9)	46 (68.7)
Duration of Initial Response		*P*=0.70	*P*=0.61	*P*=0.68
> 12 months	152 (27.4)	27 (29.7)	28 (28.9)	20 (29.9)
< 12 months	236 (42.6)	40 (44.0)	44 (45.4)	30 (44.8)
No Response (SD/PD)	166 (30.0)	24 (26.4)	25 (25.8)	17 (25.4)
Prior rituximab		*P*=0.16	*P*=0.32	*P*=0.32
Yes	416 (75.1)	63 (69.2)	69 (71.1)	47 (70.1)
Rituximab with Salvage		*P*=0.04	*P*=0.03	*P*=0.03
Yes	363 (65.5)	68 (74.7)	73 (75.3)	52 (77.6)
Transformed Lymphoma		*P*=0.29	*P*=0.18	*P*=0.06
Yes	89 (16.1)	18 (19.8)	20 (20.6)	16 (23.9)

P-values indicate comparisons of study groups with overall LY12 population. FISH: fluorescence *in situ* hybridization; IHC: immunohistochemistry; LDH: lactate dehydrogenase; DHAP: dexamethasone, high-dose cytarabine and cisplatin; GDP: gemcitabine, dexamethasone and cisplatin; R: rituximab; SD/PD: stable disease/progressive disease; ECOG: The Eastern Cooperative Oncology Group; IPI: The International Prognostic Index.

block. IHC staining was performed using a Ventana automated immunostainer (Tucson, AZ, USA) for the following proteins: CD10, BCL6, MUM1/IRF4, FOXP1, LMO2, GCET, BCL2, C-MYC, P53, and pySTAT3. Inclusion criteria for the IHC study was a diagnosis of rrDLBCL at LY12 trial entry as determined by central hematopathology review and a minimum of two out of three histo spots containing at least 200 tumor cells/histo spot. Only 91 out of the 130 (70%) samples on the TMA met these inclusion criteria for IHC staining. Protein expression was recorded in 10% increments of positive cells and cases were dichotomized by previously reported criteria: MYC (>40%), BCL2 (>70%), p53 (>30%), pySTAT3 (>50%), and COO assigned by the Hans, Choi, and tally algorithms.[11-13, 24,25,29]

In total, 97 cases had a sufficient quantity of FFPE tissue samples to successfully extract at least 500ng RNA and perform GEP to assess *BCL2, MYC, TP53*, and *STAT3* expression. Raw counts were normalized using nSolver Analysis Software v3.0. Background subtraction was performed for each sample by subtracting the mean of eight negative controls from all data points. Raw counts were further normalized to the geometric mean of nine housekeeping genes, namely *ACTB, PRL19, GAPDH* (high expressers), *PGK1, CLTC, HPRT1* (intermediate expressers) and *TBP, GUSB, ABCF1* (low expressers) to adjust possible variations in RNA quantity subjected to hybridization, between samples. At the time we performed this exploratory analysis, no clearly defined cut-off for high expression existed for digital GEP data. We arbitrarily specified the cut-off for both *MYC* and *BCL2* digital GEP to be 1.5x median prior to data analysis, as per previous reports evaluating GEP.[30-33] COO was assigned using the Lymph2Cx assay.[20,29]

Sixty-seven cases had adequate tissue for FISH. The FISH testing was completed with the use of Vysis (Abbott Park, IL, USA) break-apart probes for *MYC* and *BCL2*.

Statistical Analysis

Research personnel scoring IHC, FISH and GEP testing were blind to all clinical data. Kaplan-Meier (KM) estimate and the log-rank test were used to compare EFS and OS among different groups.[34] Multivariate Cox proportional hazards models[35] were constructed to evaluate potentially independent clinical and molecular predictors of EFS and OS. The analyses were conducted using SAS software package version 9.2,[36] and *P*-values of <0.05 were considered statistically significant.

Results

Patient Characteristics and Outcome

The 91 patients included in the IHC study, the 97 patients in the digital GEP study, and the 67 patients in the FISH study, were representative of the 554 transformed or *de novo* DLBCL patients in the LY12 trial (Table 1). Initial diagnostic tissue biopsies were used in all cases except for patients where DLBCL was a result of transformation of follicular lymphoma transformation. The median time from sample collection to study randomization was 0.93 years (interquartile range: 0.47-2.18). Figure 1 demonstrates the relationship between patients analyzed by IHC, digital GEP and FISH in a Venn diagram.

Cell-of-origin

COO was determined to be GCB in 44% of patients by Hans, 50.5% by Choi, and 50.5% by Tally IHC algorithms, and in 75% by Lymph2Cx GEP. There was a 73.2% concordance between the Hans IHC algorithm and GEP Lymph2Cx assay in determining COO, with both identifying GCB in 47.8% and non-GCB/ ABC in

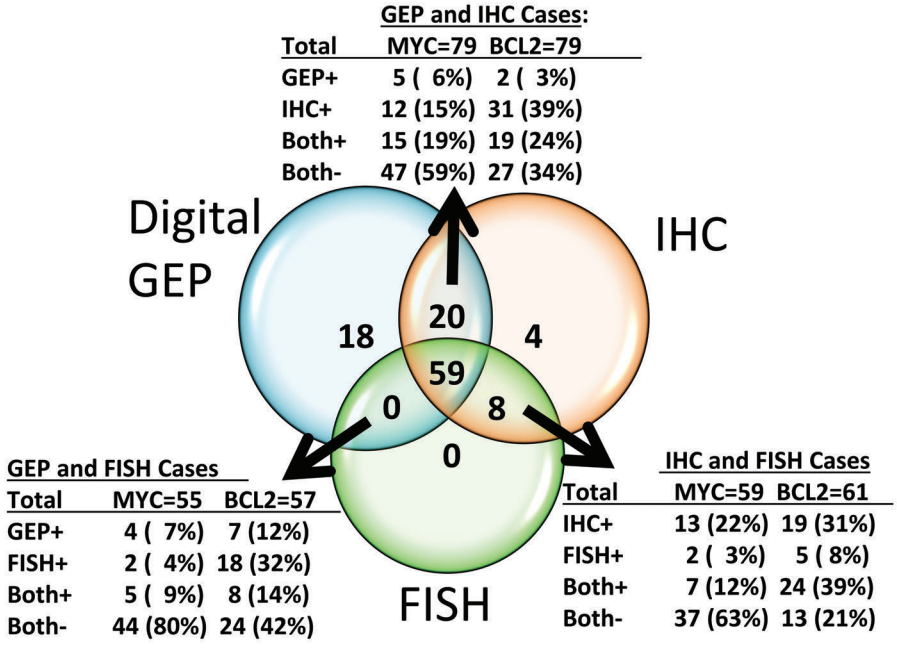

Figure 1. Relationship between IHC, digital GEP and FISH testing, and results for overlapping cases. FISH: fluorescence *in situ* hybridization; IHC: immunohistochemistry; GEP: gene expression profiling.

25.4%; while 1.5% were GCB by Hans and ABC by Lymph2Cx, and 25.3% were non-GCB by Hans and GCB by Lymph2Cx. COO was not associated with either EFS or OS, whether determined by any IHC-based algorithm (eg., Hans *P*=0.90 for EFS and *P*=0.98 for OS) or by the digital GEP Lymph2Cx assay (EFS P=0.63, OS *P*=0.25).

Biomarker Analysis

All patients

By IHC, pySTAT3 was positive in 7.7%, p53 in 19.8%, MYC in 36.3%, and BCL2 in 63.7% of cases. The overall response rate (ORR) for the 22 patients with dual protein expresser (DPE) lymphomas (MYC+/BCL2+) *vs*. single expressers (n=33) *vs*. neither MYC or BCL2 expresser (n=69) were 32.8%, 32.9% and 45.5%, respectively (*P*=0.51). MYC+ IHC was associated with lower 3-year EFS rates (10% *vs*. 42%, *P*=0.007) and OS rates (29% *vs*. 56%, *P*=0.002) compared to MYC- cases. Similarly, 3-year EFS rates were 25% *vs*. 41% (*P*=0.03) and OS rates were 37% *vs*. 63% (*P*=0.02) for BCL2+ *vs*. BCL2- cases, respectively. The 22 patients with DPE lymphomas had significantly worse 3-year EFS (0% *vs*. 40%, log-rank *P*=0.001) and OS rates (20% *vs*. 54%, log-rank *P*=0.004) relative to the other 69 patients. In addition, p53+ *vs*. p53- lymphomas had 3-year EFS rates of 11% *vs*. 36% (*P*=0.03), and 3-year OS rates of 39% *vs*. 48% (*P*=0.18). pySTAT3 was not associated with EFS (*P*=0.25) or OS (*P*=0.80). There was no interaction between treatment regimen (GDP(+/-R) or DHAP(+/-R)) and COO or MYC/BCL2 expression related to OS or EFS.

By GEP, the 1.5x median cut-off for *MYC* was 922.1 total mRNA counts (giving 24% positive cases) and for *BCL2* was 2906.6 counts (giving 25% positive cases). ORR to salvage therapy for double mRNA expressers *vs*. single expressers *vs*. neither *MYC* or *BCL2* expression were 22.2%, 21.4% and 43.3%, respectively (*P*=0.09). The nine patients with dual *MYC/BCL2* mRNA expressing lymphomas had significantly worse 3-year EFS (0% *vs*. 32%, log-rank *P*=0.007) and OS rates (0% *vs*. 45%, log-rank *P*=0.002) relative to the 88 other patients.

Because we arbitrarily chose the 70% cut-off for BCL2 expression by IHC, and the 1.5x median cut-off for digital GEP analysis, we also ran sensitivity analyses using other cut-offs. We analyzed our data using a 50% cut-point for BCL2 expression by IHC, and found that the 61 (67%)

Table 2. Univariate analysis for EFS and OS.

	OS			EFS		
Factors	HR	95%CI	*P*	HR	95%CI	*P*
Clinical Factors						
GDP(+/- R) *vs.* DHAP(+/-R)	0.868	0.501, 1.506	0.62	1.075	0.664, 1.742	0.77
Age > 60 years	1.091	0.635, 1.877	0.75	1.044	0.638, 1.710	0.86
Female *vs.* male	0.933	0.561, 1.550	0.79	0.969	0.610, 1.538	0.89
DLBCL *vs.* Transformed	1.848	0.876, 3.899	0.11	1.600	0.860, 2.978	0.14
Extranodal Sites >1 *vs*. 0-1	1.466	0.837, 2.567	0.18	0.711	0.425, 1.189	0.19
SD/PD to initial therapy	2.985	1.751, 5.102	<0.0001	2.809	4.695, 1,689	<0.0001
ECOG 2-3 *vs*. 0-1	1.197	0.568, 2.520	0.64	0.969	0.481, 1.952	0.93
B Symptoms	1.938	1.154, 3.255	0.01	1.762	1.101, 2.819	0.02
Elevated LDH	2.309	1.325, 4.032	0.003	1.608	0.996, 2.597	0.05
Stage 3-4 *vs*. 1-2	1.468	0.839, 2.571	0.18	1.277	0.775, 2.104	0.3
Digital GEP						
Lymph2Cx GCB	1.411	0.782, 2.458	0.25	1.151	0.651, 2.036	0.63
MYC	2.908	1.675, 5.045	0.0001	2.033	1.207, 3.425	0.008
BCL2	2.994	1.749, 5.128	<0.0001	2.486	1.507, 4.100	0.0004
PD1	2.214	1.236, 3.968	0.005	1.689	1.025, 2.785	0.03
PDL1	1.395	0.768, 2.532	0.26	1.242	0.737, 2.091	0.41
IHC						
Hans COO GCB	1.007	0.578, 1.754	0.98	1.033	0.630, 1.694	0.90
BCL2 > 70%	2.036	1.103, 3.757	0.02	1.764	1.051, 2.960	0.03
MYC > 40%	2.278	1.319, 3.934	0.003	1.950	1.185, 3.208	0.009
pySTAT3 > 50%	0.879	0.317, 2.438	0.85	0.559	0.203, 1.539	0.26
P53 > 50%	1.409	0.725, 2.737	0.31	1.738	0.958, 3.152	0.07
FISH						
MYC (All)	1.840	0.753, 4.494	0.18	1.243	0.555, 2.782	0.60
MYC (Double hits removed)	2.698	1.026, 7.095	0.04	1.681	0.657, 4.300	0.28
BCL2	1.162	0.604, 2.237	0.65	1.141	0.645, 2.018	0.65

CI: confidence interval; EFS: event-free survival; FISH: fluorescence *in situ* hybridization; GEP: gene expression profiling; HR: hazard ratio; IHC: immunohistochemistry; LDH: lactate dehydrogenase; OS: overall survival; ECOG: The Eastern Cooperative Oncology Group; DHAP: dexamethasone, high-dose cytarabine and cisplatin; GDP: gemcitabine, dexamethasone and cisplatin; R: rituximab; DLCBL: diffuse large B-cell lymphoma; GCB: germinal center B-cell; COO: cell-of-origin; pySTAT3: phosphoSTAT3.

patients who expressed BCL2>50% had inferior EFS (hazard ratio [HR] 1.721, 95% confidence interval [CI] 1.011-2.933, *P*=0.04) and OS (HR 1.984, 95%CI 1.058-3.717, *P*=0.03) compared to the 30 (33%) patients whose expression of BCL2 was <50%. This is a very similar result to that which we have reported using the 70% cut-point (Table 2), which demonstrated that the 58 (63.7%) patients who expressed BCL2>70% had inferior EFS (HR 1.764, 95%CI 1.051-2.960, *P*=0.03) and OS (HR 2.036, 95%CI 1.103-3.757, *P*=0.02) compared to the 33 (36.3%) patients whose expression of BCL2 was <70%. Regarding digital GEP, Figure 2 plots hazard ratios *vs.* possible thresholds and shows that the HR is predominantly over 1, suggesting that the association is robust to changes in threshold. For OS, the optimal cut-off for *MYC* was 504.0 counts (giving 29.9% positive), HR = 3.43 (95%CI 2.23, 5.86, *P*<0.0001), and for *BCL2* the optimal cut-off was 2887.5 counts (giving 26.8% positive), HR = 2.91 (95%CI 1.72, 4.95, *P*<0.0001). For EFS, the optimal cut-off for *MYC* was 803.1 counts (giving 62.1% positive), HR = 2.23 (95%CI 1.35, 3.68, *P*=0.002) and for *BCL2* the optimal cut-off was 2861.4 counts (giving 27.8% positive), HR = 2.14 (95%CI 1.32, 3.50, *P*=0.004).

By FISH, 9/63 (14.3%) patients had *MYC* rearrangement and 29/64 (45.3%) had *BCL2* rearrangement. There were three double hit lymphoma (DHL) patients by FISH analysis. All three proceeded to ASCT, with two patients relapsing at five and 27 months post-ASCT while the third is alive and relapse-free.

Transplanted patients

There were no significant differences in ORR, transplantation rate, EFS or OS between patients treated with GDP(+/-R) or DHAP(+/-R), whereas survival was associated with MYC and BCL2 protein and mRNA expression. The 3-year OS rates were 56% *vs.* 92% for patients who were transplanted for IHC-determined MYC+ *vs.* MYC- lymphomas, respectively (log-rank *P*=0.0005). The 3-year OS post-ASCT was 55% for patients who underwent ASCT for DPE lymphomas compared to 88% for patients without the DPE phenotype (log-rank *P*=0.001). Moreover, all patients with both *MYC* and *BCL2* overexpression by digital GEP relapsed.

Univariate analysis

By univariate analysis (Table 2), the only clinical factors that were significantly associated with OS and EFS were primary refractory lymphoma (no response or progressive disease to initial chemotherapy), B symptoms, and elevated LDH. MYC and BCL2 expression by IHC or digital GEP were associated with EFS and OS. However, neither *MYC* nor *BCL2* rearrangement by FISH were significantly associated with EFS or OS. As there were only three DHL patients, it was not feasible to determine whether DHL was associated with inferior EFS or OS.

Multivariate analysis

In multivariate analyses, four factors were adversely associated with EFS and OS: primary refractory DLBCL,

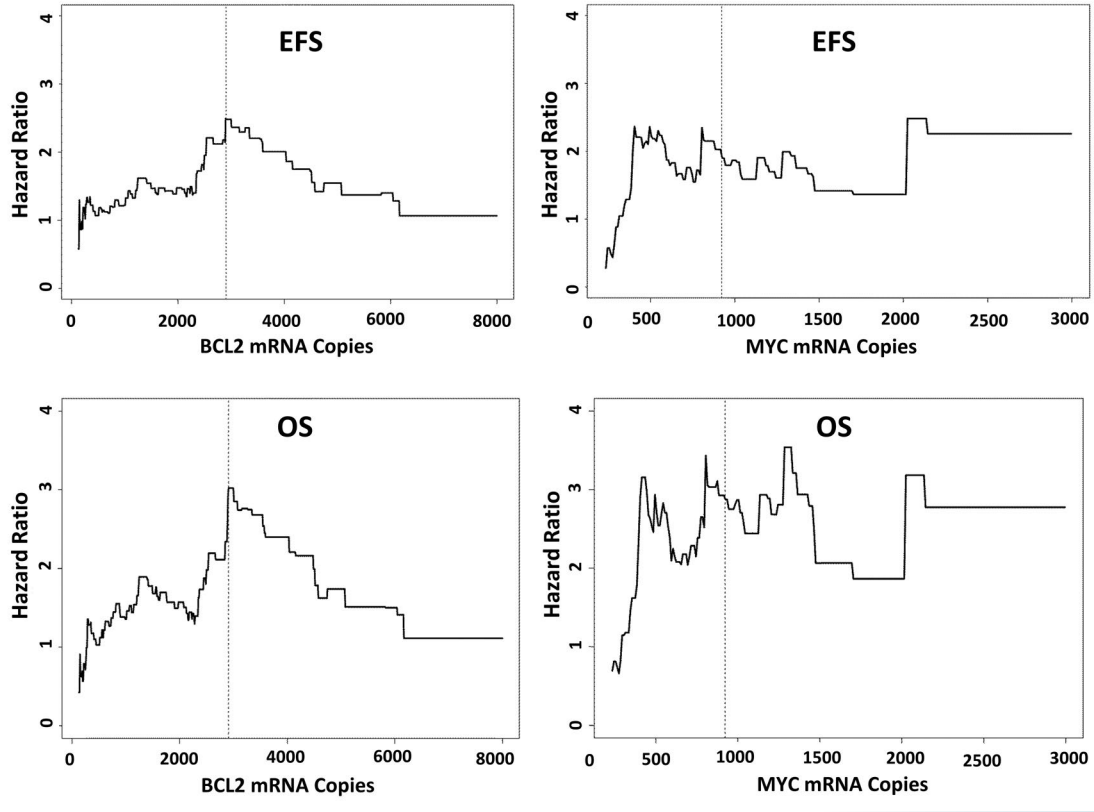

Figure 2. Hazard ratios by different thresholds of MYC and BCL2 GEP for EFS and OS. Vertical dash line indicates pre-specified 1.5x median threshold used in the analysis. EFS: event-free survival; OS: overall survival; mRNA: messenger ribonucleic acid.

elevated serum LDH at relapse, MYC expression and BCL2 expression (assessed by either IHC or digital GEP; Table 3). These four factors were associated with relatively similar HR for EFS and OS, and were therefore combined with equal weighting to create a bioclinical score that predicted ORR, EFS and OS from the initiation of salvage chemotherapy. Patients with a bioclinical score of 0-1 were considered low-risk, while those with a score or 2-4 were considered high-risk. This grouping allowed adequate numbers of patients to be analyzed within each group. Using IHC to assess MYC and BCL2, ORR was 73.5% *vs*. 45.3% (P=0.01), CR 41.2% *vs*. 30.2% (P=0.36), 3-year EFS was 55% *vs*. 16% (log-rank P<0.0001), and 3-year OS rate was 76% *vs*. 26% (log-rank P<0.0001) for the 34 patients with 0-1 factor *vs*. the 53 with 2-4 factors (see Figure 3). Similarly, using digital GEP to assess *MYC* and *BCL2* expression, ORR was 74.1% *vs*. 28.2% (P<0.0001), CR 43.1% *vs*. 23.1% (P=0.05), 3-year EFS rate was 46% *vs*. 5% (log-rank P<0.0001), and 3-year OS rate was 66% *vs*. 4% (log-rank P<0.0001) for the 58 patients with 0-1 factor *vs*. the 39 with 2-4 factors.

The same four factor model predicted EFS for the 54 patients who received ASCT. Specifically, the post-transplant 3-year EFS was 68% *vs*. 34% for 0-1 *vs*. 2-4 factors, respectively (log-rank P=0.013) assessing MYC and BCL2 by IHC, and 53% *vs*. 18% for 0=1 *vs*. 2-4 factors, respectively (log-rank P=0.008) when assessing *MYC* and *BCL2* by digital GEP.

Discussion

In the study herein, by using tissue samples and clinical data from patients with rrDLBCL enrolled in a prospective trial of salvage therapy prior to HDCT/ASCT, we were able to derive a clinical predictor of both response to salvage chemotherapy—important in the decision to proceed to transplant—and EFS and OS. Factors which were independently associated with EFS and OS in multivariate analysis included: primary refractory disease, elevated serum LDH at relapse, and MYC and BCL2 expression, assessed either by IHC or digital GEP. A bioclinical score using these four factors predicted EFS and OS. Cell-of-origin was not associated with EFS or OS regardless of whether it was assessed by IHC algorithms or the Lymph2Cx digital GEP assay.

These results are consistent with previous publications that have reported adverse ASCT outcomes for DLBCL patients with primary refractory disease, elevated LDH (either alone or as part of the IPI score), as well as MYC and BCL2 expression.[37-39] A recent retrospective study involving 331 rrDLBCL patients, of whom 132 eventually

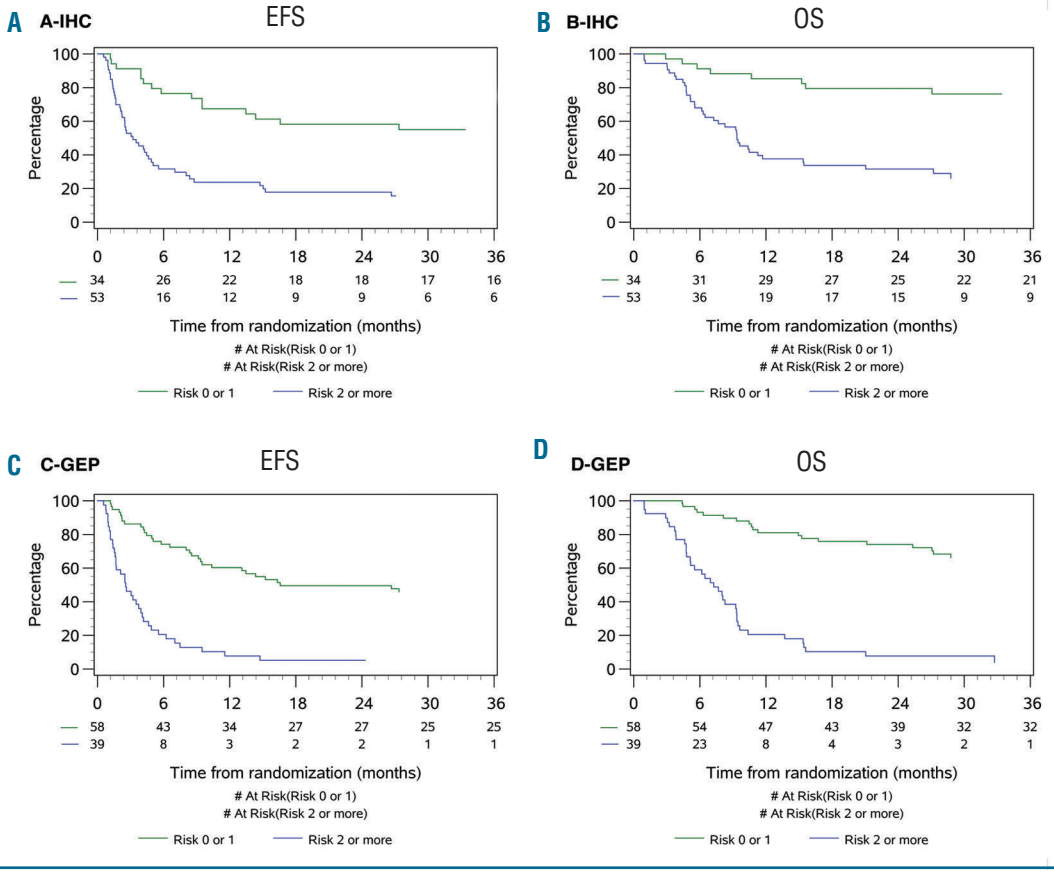

Figure 3. Outcome of rrDLBCL according to bioclinical model score comparing 0-1 factors (low-risk) *vs*. 2-4 factors (high-risk), where factors include: primary refractory disease, elevated LDH, MYC expression, and BCL2 expression. Bioclinical model assessing MYC and BCL2 by IHC (Figure 3A EFS, Figure 3B OS) or by GEP (Figure 3C EFS, Figure 3D OS). EFS: event-free survival; OS: overall survival; GEP: gene expression profiling; IHC: immunohistochemistry.

Table 3. Multivariate analyses for OS and EFS within IHC, GEP and FISH subgroups.

Factors	IHC HR	IHC 95%CI	IHC *P*	Digital GEP HR	Digital GEP 95%CI	Digital GEP *P*	FISH HR	FISH 95%CI	FISH *P*
OS									
BCL2 Expression	1.935	1.016, 3.685	0.046	3.526	1.945, 6.392	<0.0001	1.090	0.529, 2.243	0.82
MYC Expression	2.636	1.469, 4.730	0.001	2.755	1.487, 5.104	0.001	2.364	0.856, 6.528	0.10
SD/PD to Initial Therapy	3.195	1.730, 5.882	0.0002	2.899	1.605, 5.236	0.0004	2.604	1.176, 5.747	0.02
Elevated LDH at Salvage Therapy	3.484	1.818, 6.667	0.0002	2.545	1.441, 4.505	0.001	2.786	1.256, 6.173	0.01
EFS									
BCL2 Expression	1.872	1.085, 3.231	0.024	3.336	1.878, 5.925	<0.0001	1.065	0.565, 2.006	0.85
MYC Expression	2.081	1.232, 3.517	0.006	1.763	1.008, 3.086	0.047	1.710	0.699, 4.182	0.24
SD/PD to Initial Therapy	2.519	1.416, 4.484	0.002	2.299	1.330, 3.984	0.003	1.802	0.883, 3.676	0.11
Elevated LDH at Salvage Therapy	1.900	1.133, 3.175	0.015	1.976	1.163, 3.356	0.012	1.724	0.932, 3.247	0.08

CI: confidence interval; EFS; event-free survival; FISH; fluorescence *in situ* hybridization; GEP: gene expression profiling; HR: hazard ratio; IHC: immunohistochemistry; LDH: lactate dehydrogenase; OS: overall survival; SD/PD: stable disease/progressive disease.

received ASCT, reported that primary progression during rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) treatment, *MYC* translocation by FISH and IPI=3-5 at time of salvage therapy negatively affected survival, whereas COO did not.[38] Another retrospective study evaluated ASCT outcomes in 117 patients with chemotherapy-sensitive rrDLBCL, and reported inferior outcomes for those with dual protein expression (44% of patients) as well as DHL(10%).[39] Novel approaches are required for these patients.[24,38-40]

Our study had several limitations, including the small sample size of less than 100 cases with adequate tissue available for biomarker evaluation. Although this was a relatively small proportion of patients who enrolled in the LY12 clinical trial, the baseline characteristics, treatments, and outcomes of the biomarker study population were similar to the overall LY12 population of patients with aggressive B-cell lymphoma. We included transformed disease to maximize sample size as the outcomes of transformed cases were similar to DLBCL in univariate and multivariate analyses for both our IHC- and GEP-defined study populations, as well as in the overall LY12 population of 87 transformed cases and 429 rrDLBCL cases.[41] Another limitation was the fact that the biomarkers were assessed retrospectively. These assessments, however, were made blinded to all clinical factors and outcomes, and patients were treated in a prospective randomized clinical trial.

A third limitation relates to the thresholds chosen for positivity and negativity of IHC and digital GEP. Although our thresholds for IHC protein expression were consistent with published literature, several different cut-points have been reported for BCL2 positivity.[42-44] Despite our choice of the >70% cut-off for BCL2 positivity by IHC, approximately 2/3 of our cases were considered positive for BCL2 protein expression, with similar expression rates between ABC and GCB COO subtypes (*P*=0.79). This rate of BCL2 positivity using the >70% cut-off is similar to some prior studies.[42,43] Our sensitivity analysis demonstrated similar results using a >50% IHC cut-off for BCL2. Although MYC expression by IHC and GEP was fairly similar in our study, approximately 40% of our cases were positive for BCL2 by IHC but considered negative by digital GEP. This finding may be a result of our relatively high pre-specified 1.5x median threshold for GEP positivity. In order to reduce the risk of overfitting, we did not want to analyze our data according to *post hoc* optimization of multiple cut-offs for each biomarker and the endpoint of interest (eg., rates of response, ASCT, EFS or OS).[45,46] Instead we analyzed the data by a pre-specified, albeit arbitrary threshold for GEP positivity. Sensitivity analyses showed that the results using other cut-offs, including optimal cut-offs, were consistent with our pre-specified cut-off, giving HR in the same direction and the 95%CI in a similar range for EFS and OS. Our choice of 1.5x median cut-off for digital GEP worked well in predicting EFS and OS in univariate and multivariate analyses, as well as the bioclinical score, but requires confirmation in a validation cohort.

A final limitation of our study is the lack of a validation cohort, due to the modest sample size available. However, there have already been extensive publications concerning the prognostic impact of COO as well as MYC and BCL2 expression in DLBCL that generally support our findings, as discussed below. In addition, we plan to evaluate the proposed bioclinical model in our ongoing clinical trial evaluating the addition of novel agents to GDP salvage therapy for rrDLBCL.

A unique aspect of this study was the evaluation of MYC and BCL2 abnormalities by IHC, GEP, and FISH in the same patient samples. Much of the literature reporting the poor prognosis of DHLs relates to newly diagnosed DLBCL.[22,24,27,28] Analysis of our FISH cohort, however, contained only three DHL patients (4.5%), of whom two relapsed post-ASCT. In contrast, IHC identified 22 (24.2%) patients with DPE lymphoma and digital GEP identified 9 (9.3%) patients with MYC/BCL2 dual gene expression, of whom all relapsed and died after salvage therapy, while patients without dual MYC/BCL2 expression had an excellent 88% OS after transplant. If further validated, this finding may help clinicians determine who would or would not benefit from transplant.

We were unable to determine any association between COO and EFS or OS using accepted IHC algorithms or the NanoString GEP Lymph2Cx assay. It is possible that this might relate to our small sample size and low power to detect an association. Although we assessed diagnostic tissue samples for their relationship to outcome of second-line therapy, it has previously been demonstrated that biopsies

at diagnosis and relapse have similar COO phenotypes.[19] It is possible that the acquisition of other mutations or activation of pathways that are integral to drug resistance may make COO less relevant in the relapse setting. Of note, other groups have also failed to demonstrate a significant association between COO and ASCT outcomes for DLBCL.[47-49] Although the bio-CORAL study suggested that COO may be associated with the outcome of salvage therapy for rrDLBCL patients, it was only the specific interaction between rituximab - dexamethasone, cytarabine and cisplatin (R-DHAP) salvage therapy and GCB-like DLBCL (based on the Hans algorithm) that was associated with better progression-free survival (PFS), but no such association was found for the rituximab - ifosfamide, carboplatin and etoposide (R-ICE) regimen.[19] The relative greater prognostic importance of MYC and BCL2 expression over COO is also supported by the recently reported German High Grade Lymphoma Study Group (DSHNHL) retrospective study which evaluated the Nanostring Lymph2Cx COO assay in patient samples from the prospective front-line RICOVER-60 (n=326) and R-MegaCHOEP (n=88) clinical trials, and found that COO profiling failed to identify prognostic subgroups, whereas MYC/BCL2 double expression by IHC was highly predictive of poor survival.[50]

In conclusion, we combined important prognostic clinical factors with molecular biomarkers to create a novel bioclinical score that predicts outcome of salvage therapy for rrDLBCL. The model described herein identified a group of patients with 2-4 factors who had very poor 3-year EFS, whether MYC and BCL2 were assessed by IHC or by GEP. Future research is warranted to validate these findings in another dataset, and to evaluate novel agents and treatment approaches for patients who have poor prognosis rrDLBCL with dual MYC/BCL2 expression or a high-risk bioclinical score.

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