

## Concurrent Expression of MYC and BCL2 in Diffuse Large B-Cell Lymphoma Treated With Rituximab Plus Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone

Nathalie A. Johnson, Graham W. Slack, Kerry J. Savage, Joseph M. Connors, Susana Ben-Neriah, Sanja Rogic, David W. Scott, King L. Tan, Christian Steidl, Laurie H. Sehn, Wing C. Chan, Javeed Iqbal, Paul N. Meyer, Georg Lenz, George Wright, Lisa M. Rimsza, Carlo Valentino, Patrick Brunhoeber, Thomas M. Grogan, Rita M. Braziel, James R. Cook, Raymond R. Tubbs, Dennis D. Weisenburger, Elias Campo, Andreas Rosenwald, German Ott, Jan Delabie, Christina Holcroft, Elaine S. Jaffe, Louis M. Staudt, and Randy D. Gascoyne

Processed as a Rapid Communication manuscript. See accompanying editorial on page 3433 and articles on pages 3439 and 3460; listen to the podcast by Dr Gopal at [www.jco.org/podcasts](http://www.jco.org/podcasts)

Author affiliations appear at the end of this article.

Submitted December 6, 2011; accepted June 24, 2012; published online ahead of print at [www.jco.org](http://www.jco.org) on July 30, 2012.

Supported by Awards No. 019005 from the NCIC and ST-PDF-01793 from the Michael Smith Foundation for Health Research for research fellow of the Terry Fox Foundation and by an award from the "Fonds de Recherche en Santé du Québec (N.A.J.); by the NCIC and Grant No. 019001 from the Terry Fox Foundation (J.M.C., R.D.G.); and by Grant No. TGT-53912 from the Terry Fox Foundation Strategic Health Research Training Program in Cancer Research at Canadian Institutes of Health Research (D.W.S., K.L.T.). Members of the Lymphoma/Leukemia Molecular Profiling Project are supported by Strategic Partnering to Evaluate Cancer Signatures Grant No. U01-CA-114778 from the National Cancer Institute.

N.A.J. and G.W.S. contributed equally to this work.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Corresponding author: Randy D. Gascoyne, MD, Pathology and Laboratory Medicine, British Columbia Cancer Agency and the BC Cancer Research Centre, 675 West 10th Ave, Room 5-113, Vancouver, BC, Canada V5Z 1L3; e-mail: [rgascoyn@bccancer.bc.ca](mailto:rgascoyn@bccancer.bc.ca).

© 2012 by American Society of Clinical Oncology

0732-183X/12/3028-3452/\$20.00

DOI: 10.1200/JCO.2011.41.0985

### ABSTRACT

#### Purpose

Diffuse large B-cell lymphoma (DLBCL) is curable in 60% of patients treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). *MYC* translocations, with or without *BCL2* translocations, have been associated with inferior survival in DLBCL. We investigated whether expression of *MYC* protein, with or without *BCL2* protein expression, could risk-stratify patients at diagnosis.

#### Patients and Methods

We determined the correlation between presence of *MYC* and *BCL2* proteins by immunohistochemistry (IHC) with survival in two independent cohorts of patients with DLBCL treated with R-CHOP. We further determined if *MYC* protein expression correlated with high *MYC* mRNA and/or presence of *MYC* translocation.

#### Results

In the training cohort ( $n = 167$ ), *MYC* and *BCL2* proteins were detected in 29% and 44% of patients, respectively. Concurrent expression (*MYC* positive/*BCL2* positive) was present in 21% of patients. *MYC* protein correlated with presence of high *MYC* mRNA and *MYC* translocation (both  $P < .001$ ), but the latter was less frequent (both 11%). *MYC* protein expression was only associated with inferior overall and progression-free survival when *BCL2* protein was coexpressed ( $P < .001$ ). Importantly, the poor prognostic effect of *MYC* positive/*BCL2* positive was validated in an independent cohort of 140 patients with DLBCL and remained significant ( $P < .05$ ) after adjusting for presence of high-risk features in a multivariable model that included elevated international prognostic index score, activated B-cell molecular subtype, and presence of concurrent *MYC* and *BCL2* translocations.

#### Conclusion

Assessment of *MYC* and *BCL2* expression by IHC represents a robust, rapid, and inexpensive approach to risk-stratify patients with DLBCL at diagnosis.

*J Clin Oncol* 30:3452-3459. © 2012 by American Society of Clinical Oncology

### INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin's lymphoma and is curable in more than 60% of patients treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP).<sup>1</sup> The best available clinical tool to risk-stratify patients with DLBCL at diagnosis is the International Prognostic Index (IPI); however, there remains marked heterogeneity in

clinical outcomes within each risk group, and IPI variables do not provide insight into the underlying tumor biology. Gene expression profiling (GEP) can group DLBCL into prognostically different molecular subtypes based on cell-of-origin (COO) gene signatures, where the activated B-cell (ABC) type is associated with inferior overall survival (OS) compared with the germinal center B-cell (GCB) type.<sup>2,3</sup> GEP is not available in most clinical laboratories; thus, immunohistochemical

algorithms, such as the one proposed by Choi et al,<sup>4</sup> have been developed assigning a COO subtype based on the expression of COO-related proteins.<sup>5,6</sup> Unfortunately, the accuracy with which these algorithms correctly classify COO subtype or predict OS is variable among laboratories.<sup>4,6,7</sup>

Alterations in oncogenes and tumor suppressor genes can drive the pathogenesis of DLBCL.<sup>8,9</sup> Two such oncogenes are *MYC* and *BCL2*, key regulators of cellular proliferation and apoptosis, respectively.<sup>10,11</sup> Deregulation of *MYC* and *BCL2* can result from chromosomal translocation or gene amplification, but it may also occur by other mechanisms, such as transcriptional upregulation downstream of NF $\kappa$ B pathway signaling.<sup>10,12,13</sup>

The presence of *MYC* translocation and high *MYC* mRNA expression have recently been associated with poor OS in patients with DLBCL treated with R-CHOP, raising questions about optimal management of these high-risk patients.<sup>14-16</sup> However, many of these patients with *MYC*-positive DLBCL also coexpress high levels of *BCL2* protein, which may be a confounding factor in this disease, given that the presence of concurrent *MYC* and *BCL2* translocations—so-called double hits (DHITs)—are associated with a dismal outcome despite high-dose chemotherapy.<sup>14-19</sup> Fluorescence in situ hybridization (FISH) has been useful at identifying *MYC* translocations but has failed to identify altered *MYC* expression by other mechanisms and is not available in all clinical laboratories. Recently, a novel monoclonal antibody that targets the N-terminus of the *MYC* protein was shown to provide sensitive and specific staining of nuclear *MYC* in paraffin embedded tissue, including DLBCL.<sup>20-22</sup>

Herein, we demonstrate that *MYC* protein expression by IHC represents a rapid and inexpensive marker to identify *MYC* overexpression in DLBCL, including patients harboring *MYC* translocations, and that the prognostic significance of *MYC* deregulation in R-CHOP-treated patients with DLBCL depends on its coexpression with *BCL2* protein.

## PATIENTS AND METHODS

### Patient Population

We used pretreatment tumor biopsies taken from two independent cohorts of patients diagnosed with de novo DLBCL according to WHO classification (2008) criteria.<sup>1</sup> Patients were initially selected because they were linked to clinical information, including baseline characteristics and outcome, were HIV negative, and were treated with curative intent with R-CHOP therapy (with or without radiation). Ethical approval was granted by the research ethics board of each institution, in accordance with the Declaration of Helsinki.

The training set consisted of 167 patients who were further selected based on the availability of both fresh frozen and formalin-fixed paraffin-embedded (FFPE) tissue, provided from 10 international institutions. A consensus diagnosis of DLBCL was confirmed by a panel of expert pathologists. A subset of these patients were previously reported by Lenz et al<sup>3</sup> (n = 158), Savage et al<sup>14</sup> (n = 49), Iqbal et al<sup>23</sup> (n = 167), and Choi et al<sup>4</sup> (n = 68). DLBCL molecular subtype (GCB, ABC, and unclassifiable) and molecular Burkitt's lymphoma, if present in DLBCL patient cases with high *MYC* expression, were assigned by GEP according to previously published protocols.<sup>3,24,25</sup>

The validation set consisted of 140 patients from the British Columbia Cancer Agency (BCCA) who were selected based on availability of FFPE tissue only. Nine patients were included in the study by Savage et al<sup>14</sup>; COO for these patients was assigned by IHC according to the Choi et al<sup>4</sup> algorithm.

### IHC Analysis

Cores of FFPE tissue were used to construct tissue microarrays at each participating institution. In both data sets, staining was performed on the

Ventana platform (Roche, Basel, Switzerland) using routine staining protocols and the following antibodies: *BCL2*, clone 124 (Dako, Carpinteria, CA); *MYC*, Y69 (Epitomics, Burlingame, CA) and Ki67 (Dako; Appendix, online only). Protein expression was recorded in 10% increments as the percentage of positive tumor cells. Training set data were analyzed using X-Tile statistical software (<http://www.tissuearray.org/rimmlab>) to determine the optimum survival cut points for dichotomizing expression of *MYC* protein ( $\geq 40\%$ ), *BCL2* protein ( $\geq 50\%$ ), and Ki67 index ( $\geq 90\%$ ).<sup>26</sup> These cut points correspond to the maximum  $\chi^2$  value of the Mantel-Cox test for OS between groups above and below the cut-point threshold.<sup>26</sup> These same cut points were then carried through to the validation set.

The reproducibility of *BCL2* and *MYC* protein expression by IHC was determined by comparing the results obtained by three different pathologists. In the training set, *BCL2* expression in approximately half of the patients was scored by three pathologists at the University of Nebraska Medical Center (W.C.C., P.N.M., D.D.W.), with no disagreement; thus, the latter half were evaluated by a single pathologist (P.N.M.).<sup>23</sup> *MYC* and Ki67 expression were determined by a single pathologist (G.W.S.) at the BCCA. In the validation set, three pathologists at the BCCA (G.W.S., K.L.T., R.D.G.) scored patient cases for *BCL2*, *MYC*, and Ki67 protein expression using the same antibodies and thresholds described for the training set. Concordance was achieved when all three pathologists assigned the same positive or negative expression value for a patient. Discordant patient cases were evaluated by all three pathologists at a multiheaded microscope to reach a consensus score.

### FISH

Translocations involving *BCL2* and *MYC* were identified by interphase FISH in FFPE tissue in tissue microarrays from both data sets using commercial dual-color break-apart probes from Abbott Molecular (Abbott Park, IL) according to previously described methods.<sup>14</sup> Patient cases with break-apart signals in  $> 5\%$  of nuclei were considered positive for the presence of a translocation. In the training and validation cohorts, FISH experiments were successful in 167 and 123 patient cases using the *MYC* probe and in 157 and 120 using the *BCL2* probe, respectively.

### GEP

GEP was performed on fresh frozen tissue from the training set using Affymetrix HG U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA).<sup>3</sup> *MYC* mRNA expression was determined using log<sub>2</sub> normalized expression values of probe set 202431\_s\_at and dichotomized into low versus high expression using a cutoff threshold determined by X-Tile (high,  $> 9.4$ ).<sup>26</sup>

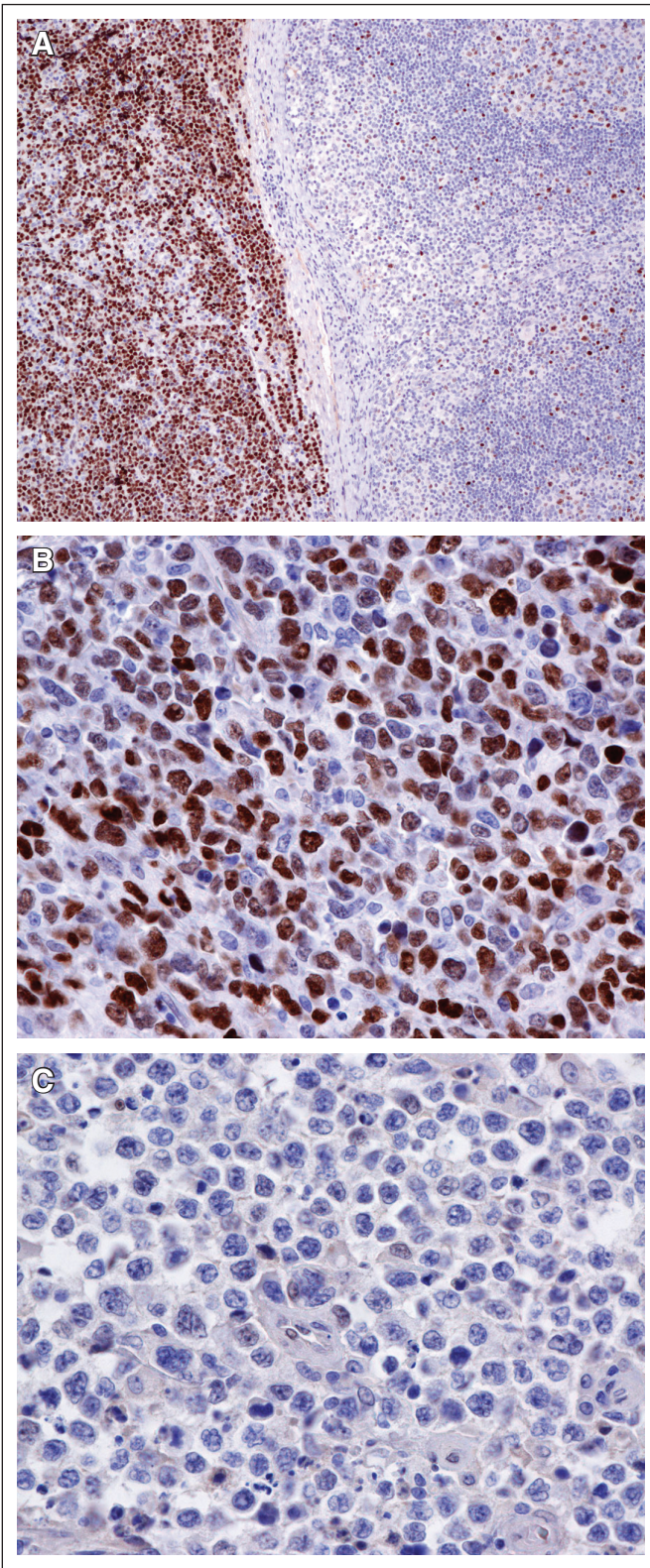
### Statistical Analysis

Progression-free survival (PFS; PFS event, progression or death resulting from any cause) and OS (OS event, death resulting from any cause) were measured from the date of pathologic diagnosis and estimated using the Kaplan-Meier method. Differences were assessed using the log-rank test. The Cox proportional hazards model was used to determine hazard ratios (HRs) and CIs and whether variables were independent of the IPI by multivariate analysis. The  $\chi^2$  test, Fisher's exact test, and Pearson correlation were used to determine association and correlation between variables. These statistical analyses were performed using SPSS (version 20.0; SPSS, Chicago, IL) and STATA software (version 8.2; STATA, College Station, TX).

## RESULTS

### *MYC* and *BCL2* Expression in DLBCL

Within the training set, *MYC* protein was expressed in one third of patients with DLBCL (n = 48 of 167; 29%). In contrast, *MYC* translocations and high *MYC* mRNA expression were detected in only 18 (11%) of 167 and 19 (11%) of 167 patients, respectively, with nine overlapping both groups. *MYC* protein expression was exclusively nuclear in all cases (Figs 1A to 1C) and was reproducible among pathologists (concordance 94%). Incidence of *MYC* protein expression (52 of 140; 37%) and *MYC* translocation (16 of 123; 13%) in the



**Fig 1.** Representative immunohistochemical analysis of MYC protein expression in diffuse large B-cell lymphoma (DLBCL). (A) Control: partial lymph node involvement by Burkitt's lymphoma (left) and follicular hyperplasia (right); there is bright nuclear staining of MYC protein in Burkitt's lymphoma cells, compared with < 5% of benign germinal center B cells stained for MYC (right). (B) MYC protein-positive DLBCL. (C) MYC protein-negative DLBCL.

validation set was similar to that in the training set (Table 1). When looking at MYC protein expression in the combined data sets, there was a wide distribution of MYC protein expression across samples (Appendix Fig A1, online only). The percentage of cells expressing MYC protein was significantly greater in patient cases with translocations than without translocations (mean percent positive cells, 61%; range, 20 to 100 v 29%; range, 0 to 100, respectively;  $P < .001$ ). Although MYC protein expression correlated with a high proliferation rate ( $r = 0.41$ ;  $P < .001$ ), MYC protein expression, at our predetermined 40% threshold, was more sensitive than Ki67 at identifying patients with MYC translocations (25 of 34; 74% v 7 of 34; 21%). Alterations involving MYC were present in both the ABC and GCB subtypes of DLBCL, whether defined by GEP (training set) or by IHC (validation set; Table 2).

BCL2 protein expression was associated with the ABC subtype, and BCL2 translocation was associated with the GCB subtype ( $P = .001$ ; Table 2). Both were more common in the validation cohort, which included older patients ( $P < .05$ ; Table 1). Indeed, there was an age-related increase in the incidence of BCL2 protein expression, BCL2 translocation, and ABC subtype, but not MYC alterations (Appendix Fig A2, online only). Importantly, BCL2 protein expression by IHC was reproducible among pathologists, with a concordance of more than 91%.

#### Factors Associated With Clinical Outcome

In univariate analysis, MYC protein expression and MYC translocation, alone, were not significantly associated with survival in the training set ( $P > .05$ ). Factors that were associated with OS and PFS included elevated IPI score, ABC subtype, high MYC mRNA expression, and BCL2 protein expression (Appendix Table A1, online only). Next, we determined if BCL2 protein expression affected prognosis of MYC deregulation and whether there was an interaction between MYC and BCL2 variables. Presence of MYC translocation, high MYC mRNA expression, or high MYC protein expression, hereafter referred to as MYC positive, was only associated with inferior OS and PFS when BCL2 protein was coexpressed (BCL2 positive; Figs 2A to 2C;  $P < .001$ ). Indeed, the interaction between these variables suggested that the negative prognostic impact of MYC and BCL2 was amplified when both variables were present, an effect that was consistent across platforms (protein, mRNA, or translocation; Appendix Table A1, online only). For example, the OS HR for coexpression of MYC-positive/BCL2-positive proteins was 3.2, compared with 0.47 for the MYC-positive/BCL2-negative group and 1.6 for the MYC-negative/BCL2-positive group ( $P = .001$ ,  $P = .11$ , and  $P = .17$ , respectively). In the validation cohort, coexpression of MYC-positive/BCL2-positive proteins was also associated with inferior OS (HR, 1.6;  $P = .17$ ), but there was no difference in outcome in the remaining patients (Appendix Fig A3, online only). Samples were then stratified into a high-risk group, defined by the presence of MYC-positive/BCL2-positive protein coexpression, and low-risk group, comprising the remaining patients. Presence of MYC-positive/BCL2-positive protein expression was associated with significantly inferior OS and PFS in both training and validation DLBCL cohorts (Figs 3A and 3B;  $P < .05$ ). Importantly, the negative prognostic impact of MYC-positive/BCL2-positive status on OS persisted even after adjusting for IPI and COO in a Cox multivariate model ( $P \leq .05$ ; Appendix Table A2, online only).

**Table 1.** Clinical Characteristics of Two Cohorts of Patients With DLBCL Treated With R-CHOP

Characteristic	All Patients (N = 307)		Training Cohort (n = 167)		Validation Cohort (n = 140)		P*
	No.	%	No.	%	No.	%	
Age, years							
Median	63		62		65		
Range	17-92		17-92		19-90		
> 60	179 of 307	58	85 of 167	51	94 of 140	67	<b>.004</b>
Ann Arbor stage > II	162 of 296	55	81 of 161	50	81 of 135	60	.107
LDH > upper limit of normal	126 of 297	42	67 of 167	40	59 of 130	45	.364
Extranodal sites ≥ two	50 of 261	19	19 of 126	15	31 of 135	23	.107
ECOG PS > 1†	78 of 282	28	36 of 148	24	42 of 134	31	.190
IPI score‡							<b>.021</b>
0 or 1	114 of 288	40	72 of 154	47	42 of 134	31	
2	76 of 288	26	41 of 154	27	35 of 134	26	
3	54 of 288	19	22 of 154	14	32 of 134	24	
4 or 5	44 of 288	15	19 of 154	12	25 of 134	19	
R-CHOP21			159	95	140	100	
R-CHOP14			8	5			.759
Radiation			37	22	29	21	
Cell-of-origin subtype			GEP		IHC§		
ABC	139 of 306	45	70 of 167	42	69 of 139	50	.178
GCB	144 of 306	47	74 of 167	44	70 of 139	50	
Unclassified	21 of 306	7	21 of 167	13			
Molecular Burkitt's lymphoma	2 of 306	1	2 of 167	1			
MYC							
MYC translocation	34 of 290	12	18 of 167	11	16 of 123	13	.561
High mRNA expression			19 of 167	11			
High MYC protein expression	100 of 307	33	48 of 167	29	52 of 140	37	.094
BCL2							
BCL2 translocation	68 of 287	24	29 of 157	18	39 of 130	30	<b>.022</b>
High BCL2 protein expression	160 of 304	53	73 of 164	44	87 of 140	62	<b>.002</b>
Median follow-up time, years¶			3.5		4.7		.503
5-year OS				64		62	

NOTE. Bold font indicates significance.

Abbreviations: ABC, activated B-cell-like; DLBCL, diffuse large B-cell lymphoma; ECOG PS, Eastern Cooperative Oncology Group performance status; GCB, germinal center B-cell-like; GEP, gene expression profiling; IHC, immunohistochemistry; IPI, International Prognostic Index; LDH, lactate dehydrogenase; OS, overall survival; R-CHOP, rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone; R-CHOP14, R-CHOP 14-day cycle; R-CHOP21, R-CHOP 21-day cycle.

\*P values are for comparison between training and validation cohorts.

†ECOG PS ranges from 0 to 4, where higher score indicates greater degree of impairment.

‡IPI score ranges from 0 to 5, with 0 indicating absence of prognostic factors and 5 indicating presence of all prognostic factors; IPI score was not calculated if more than one variable was unavailable.

§IHC algorithm according to Choi et al.<sup>4</sup>

||Unclassified indicates gene expression profiles are intermediate between ABC and GCB.

¶Median follow-up time for living patients was 3.5 years (range, 0.52 to 11.3 years) and 4.7 years (range, 1.0 to 8.0 years) for training and validation sets, respectively.

### Concurrent Translocation of MYC and BCL2

There were 14 patient cases in the combined data set (14 of 290; 5%) fitting the diagnostic criteria of DLBCL but with concurrent translocation of *MYC* and *BCL2* (ie, DHIT). Given this low incidence, both data sets were pooled for subsequent analyses. These patients had more adverse clinical risk factors, including higher levels of lactate dehydrogenase, worse performance status, and higher IPI scores than other patients with DLBCL ( $P < .05$ ; Appendix Table A3, online only). DHIT samples expressed BCL2 protein (13 of 13); 10 (71%) of 14 samples expressed MYC protein, but only one (7%) of 14 had Ki67 > 90%. As expected, the clinical outcome of patients with DLBCL with DHIT status treated with R-CHOP in this study was extremely poor, with 5-year OS and PFS rates of 27% and 18%, respectively (Fig 3C). However, the outcome of the remaining MYC-positive/BCL2-positive patients (excluding those with DHIT status) was also poor

compared with outcome among those who did not express MYC-positive/BCL2-positive proteins (Fig 3C; 5-year OS and PFS, 36% and 32% v 71% and 65%, respectively;  $P < .05$ ). Interestingly, two of the three long-term survivors in the DHIT group had < 40% cells in their biopsy expressing MYC protein, suggesting that this may have prognostic relevance, analogous to what has been previously reported for BCL2 protein expression in DHITs,<sup>19</sup> but too few patient cases preclude any meaningful conclusions.

### DISCUSSION

Overexpression of MYC protein occurs in 33% of patient cases with DLBCL, of which only one third can be explained by the presence of MYC translocation. That MYC protein is expressed in both the GCB

**Table 2.** Differences in *MYC* and *BCL2* Alterations According to Cell-of-Origin Subtype in Two Cohorts of Patients With DLBCL

Cohort	ABC		GCB		Unclassified*		mBL		P†
	No.	%	No.	%	No.	%	No.	%	
<b>MYC</b>									
<i>MYC</i> translocation									
Training‡	6 of 70	9	10 of 74	14	1 of 21	5	1 of 2	50	.346
Validation§	6 of 62	10	10 of 61	16					.268
High mRNA expression									
Training	10 of 70	14	5 of 74	7	2 of 21	10	2 of 2	100	.139
<i>MYC</i> protein expression									
Training	24 of 70	34	17 of 74	23	6 of 21	29	1 of 2	50	.399
Validation	<b>35 of 69</b>	<b>51</b>	17 of 70	24					<b>.001</b>
<b>BCL2</b>									
<i>BCL2</i> translocation									
Training	4 of 70	6	<b>23 of 74</b>	<b>31</b>	2 of 11	10	0	0	< .001
Validation	11 of 65	17	<b>28 of 65</b>	<b>43</b>					<b>.001</b>
<i>BCL2</i> protein expression									
Training	<b>44 of 70</b>	<b>63</b>	22 of 74	30	7 of 21	35	0	0	< .001
Validation	<b>53 of 69</b>	<b>77</b>	34 of 70	49					<b>.001</b>
DHIT  ¶	5 of 14	36	9 of 14	64					
<i>MYC</i> and <i>BCL2</i> protein expression¶	42 of 55	76	13 of 55	24					< .001
Absence of concurrent <i>MYC</i> and <i>BCL2</i> protein expression or DHIT	92 of 235	39	143 of 235	61					

NOTE. Bold font indicates significance.

Abbreviations: ABC, activated B-cell lymphoma; DHIT, double-hit lymphoma; DLBCL, diffuse large B-cell lymphoma; GCB, germinal center B-cell lymphoma; GEP, gene expression profiling; mBL, molecular Burkitt's lymphoma.

\*Unclassified indicates gene expression profiles are intermediate between ABC and GCB.

†All *P* values refer to comparison between GCB and ABC subtypes.

‡In training set, cell-of-origin distinctions were assigned according to GEP and signatures previously described by Wright et al<sup>25</sup> and Dave et al.<sup>24</sup>

§In validation set, cell-of-origin distinctions were assigned according to immunohistochemical algorithm reported by Choi et al.<sup>4</sup>

||DHIT refers to presence of concurrent *MYC* and *BCL2* translocations.

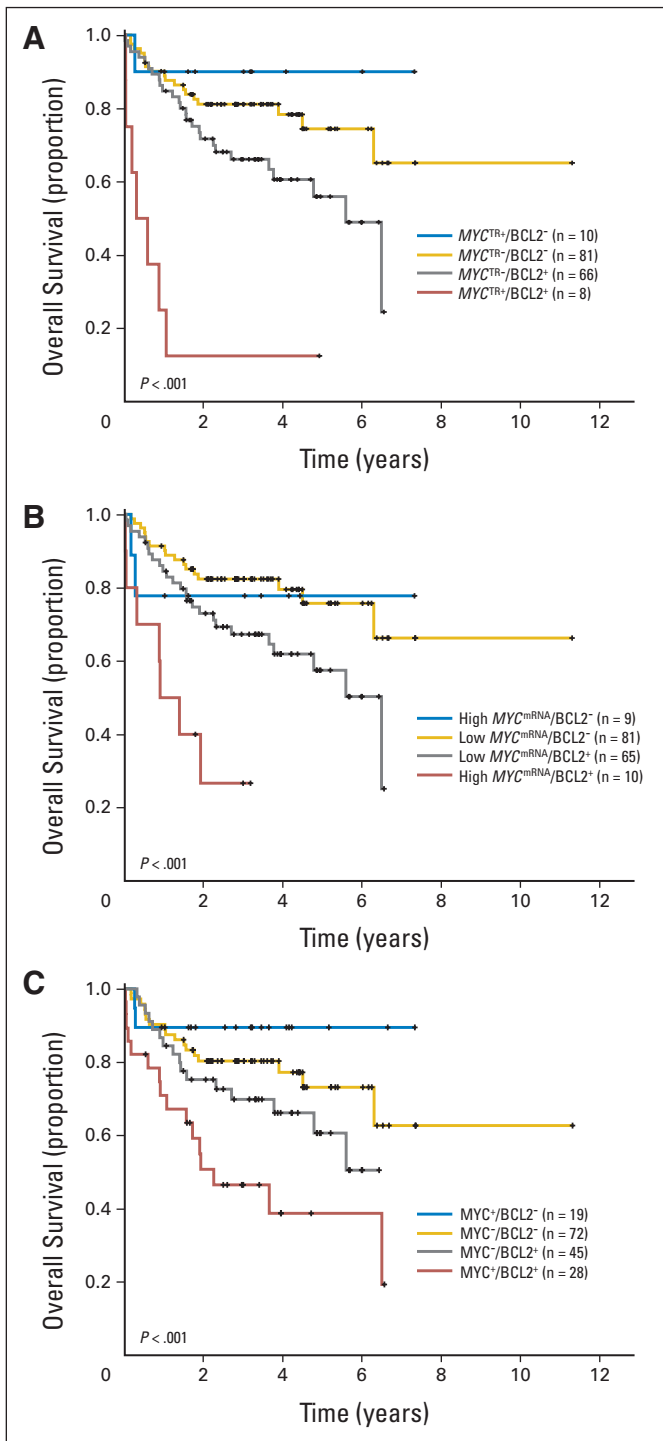
¶Frequencies and *P* values refer to differences between molecular subtypes in combined data sets (training and validation), where patients were stratified according to presence of DHIT or presence of concurrent *MYC* and *BCL2* proteins, which excluded DHITs. Cell-of-origin data were not available in one of 307 patient cases, and *BCL2* protein was not available in three of 307 patient cases, one of which was DHIT.

and ABC subtypes suggests an underlying biologic pathway independent of COO subtype. In addition to promoting cell-cycle progression, *MYC* plays an important role in metabolism, protein synthesis, stem-cell renewal, and mRNA regulation and can induce apoptosis by increasing p53 expression or amplifying apoptotic signaling pathways.<sup>27-30</sup> Given this broad range of biologic activity, it is not surprising that deregulation of *MYC* is oncogenic.<sup>10</sup> *MYC* protein expression has also been found to occur as a consequence of other genomic events (eg, inactivating *p53*), increased protein stability, activation of upstream signaling pathways (eg, NFκB), desensitization to inhibitory cytokines, and loss of host immunity.<sup>20,31,32</sup> Given that many *MYC*-positive samples did not have a high proliferation rate in this study, these nonproliferative functions of *MYC* may contribute to the clinical and biologic attributes of DLBCL, possibly by inducing further DNA damage and genomic instability.<sup>33</sup> Thus, *MYC* protein expression by IHC in DLBCL may represent a final integrator of all or most of the mechanisms that underlie *MYC* deregulation.

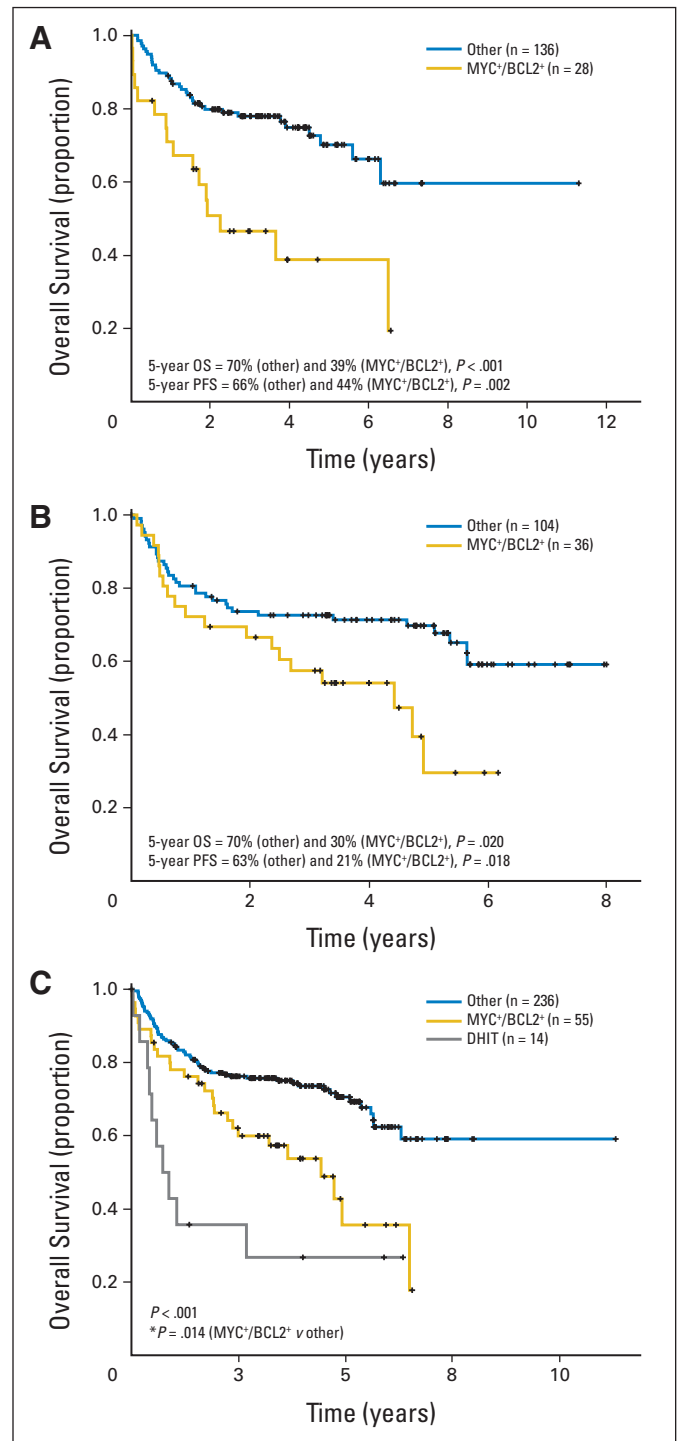
We found *MYC* and *BCL2* IHC interpretation to be robust, reproducible, and largely unaffected by the vagaries introduced by differing fixation techniques used by the 10 institutions participating in this study. The cutoff scores for many biomarkers have often been arbitrary; in the case of *BCL2*, they have ranged from > 10% to > 75%, making comparison among studies difficult.<sup>34,35</sup> As such, we defined our thresholds based on rational statistical methods in accordance with recommended guidelines.<sup>36</sup> Our *BCL2* threshold com-

pares favorably with that defined by Tzankov et al,<sup>34</sup> who used receiver operator curves to define optimal cutoff scores, but it is lower than the ≥ 75% threshold reported by Salles et al,<sup>35</sup> who used a statistical approach similar to that of our study. The latter study investigated IHC markers in 2,451 DLBCL samples within the Lunenburg Lymphoma Biomarker Consortium, many of which were derived from young patients enrolled onto clinical trials, including studies evaluating R-CHOP administered every 14 days.<sup>35</sup> These data suggest that age and treatment may be important factors to consider when defining the optimal thresholds of biomarkers in DLBCL. Indeed, we and others have shown that there are age-related changes in tumor biology and that treatment, such as rituximab, can influence the prognostic impact of several biomarkers in DLBCL, including *BCL2*.<sup>16,37-42</sup>

We also demonstrated that *BCL2* protein expression is the main determinant of clinical outcome in *MYC*-positive DLBCL. This biologic effect was robust across different platforms (IHC, GEP, and FISH) and was validated in a second independent cohort, which was representative of a population-based registry, suggesting that this biomarker may be relevant even in older patients.<sup>16</sup> Furthermore, coexpression of *MYC* and *BCL2* proteins by IHC remained significant after adjusting for IPI, COO, and presence of DHIT. This is in keeping with the known biologic function of *BCL2*, which is a potent inhibitor of apoptosis and has been clearly shown to mediate chemotherapy resistance in *MYC*-positive lymphoma murine models.<sup>43</sup> Although previous studies have demonstrated a negative prognostic effect of *MYC*



**Fig 2.** Overall survival (OS) of patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone based on alterations in MYC and BCL2 in the training set. Kaplan-Meier curves represent OS according to (A) presence of MYC translocation (TR) and BCL2 protein expression (BCL2+), (B) presence of high MYC mRNA expression and BCL2 protein expression, and (C) presence of MYC and BCL2 protein expression. Log-rank  $P < .001$  for both OS and progression-free survival. Total evaluable patients for the analyses: (A)  $n = 165$ , (B)  $n = 165$ , and (C)  $n = 164$ .



**Fig 3.** Overall (OS) and progression-free survival (PFS) of patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone according to presence of concurrent expression of MYC and BCL2 proteins (MYC<sup>+</sup>/BCL2<sup>+</sup>) and/or presence of concurrent MYC and BCL2 translocations. Kaplan-Meier curves represent OS according to presence of MYC<sup>+</sup>/BCL2<sup>+</sup> in (A) the training cohort ( $n = 164$ ) and (B) validation cohort ( $n = 140$ ). (C) Both training and validation cohorts were combined because of the low frequency of double hits (DHITs). Patients were stratified according to presence of DHIT or MYC<sup>+</sup>/BCL2<sup>+</sup> excluding DHITs. Four DHIT patient cases had no MYC protein expression; one had a missing value for BCL2 protein expression.

translocation in DLBCL, a majority of patients' samples were BCL2 protein positive,<sup>14,16</sup> and it is unknown whether those with *MYC* translocations, but lacking BCL2 expression, would share a similar fate. This is an important question if patients with *MYC*-positive DLBCL are to be considered for more aggressive therapy. Unfortunately, there are no data to support the use of any one regimen for the treatment of *MYC*-positive/*BCL2*-positive DLBCL at this time. The results from the MRC/NCRI (Medical Research Council/National Cancer Research Institute) LY10 trial and a recent report from Snurderl et al<sup>18</sup> did not demonstrate a survival advantage in treating patients with DHIT lymphoma with high-dose regimens, most of which would not be well tolerated in older patients.<sup>44,18</sup> The probable role that BCL2 has in this disease suggests that *MYC*-positive/*BCL2*-positive tumors may be amenable to chemotherapy regimens that include drugs targeting BCL2, such as BH3 mimetics, a strategy that has been shown to cure a subset of murine *MYC*-positive/*BCL2*-positive lymphomas.<sup>45-47</sup>

The primary aim of this study was to determine the role of *MYC* protein as a prognostic biomarker in DLBCL, not as a potential screening test to identify patients harboring *MYC* translocation. Our results confirm those of two recent studies demonstrating the excellent reproducibility of *MYC* protein by IHC among pathologists, the high correlation with presence of *MYC* translocation, and the weak correlation with Ki67. *MYC* protein by IHC in our study had a sensitivity of 74%, which was lower than the 100% reported by the other studies that evaluated it as a screening test for identifying patients with *MYC* translocations. Our study used a lower threshold (40% v 50%<sup>21</sup> and 70%<sup>22</sup>), which was set based on the relationship between *MYC* protein and survival, not the presence of a translocation. Furthermore, the study by Kluk et al<sup>21</sup> included too few patient cases to meaningfully address this issue (n = five translocations of 56 samples of de novo DLBCL), and that by Green et al<sup>22</sup> included patient cases of Burkitt's lymphoma, which raised the baseline incidence of *MYC* translocations to 15%, a level higher than that observed in de novo DLBCL. Taken together, patients with *MYC* translocation are likely to express *MYC* protein. More importantly, our data suggest that *MYC* protein expression is more common than *MYC* translocation, and the *MYC*-positive/*BCL2*-positive immunophenotype is itself a powerful predictor of survival, independent of presence of *MYC* translocation.

In conclusion, this study has identified a group of clinically high-risk patients with DLBCL who coexpress *MYC* and *BCL2* proteins, a clinical scenario that is more common than patients with lymphomas harboring the DHIT genotype (18% v 5%, respectively). Importantly, these biomarkers retained their prognostic significance in an independent cohort that consisted of patients from a population-based registry. Unlike with FISH, assessment of *MYC* and *BCL2* protein

expression by IHC represents a rapid, inexpensive, and reproducible technique that could be adopted by pathologists in most clinical centers. These promising results need to be validated prospectively in larger cohorts, using standardized staining and scoring methodologies, before implementation as prognostic biomarkers in clinical practice. Thus, *MYC* and *BCL2* represent relevant biomarkers that should be tested in the context of clinical trials such that more effective therapies can be offered to these high-risk patients.

#### AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

*Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.*

**Employment or Leadership Position:** Patrick Brunhoeber, Ventana Medical Systems (C) **Consultant or Advisory Role:** None **Stock Ownership:** None **Honoraria:** None **Research Funding:** Kerry J. Savage, Hoffmann-La Roche (Roche Canada); Joseph M. Connors, Hoffmann-La Roche (Roche Canada); Lisa M. Rimsza, Ventana Medical Systems; Randy D. Gascoyne, Hoffmann-La Roche (Roche Canada) **Expert Testimony:** None **Other Remuneration:** Lisa M. Rimsza, Ventana Medical Systems

#### AUTHOR CONTRIBUTIONS

**Conception and design:** Nathalie A. Johnson, Randy D. Gascoyne **Provision of study materials or patients:** Graham W. Slack, Joseph M. Connors, Wing C. Chan, Lisa M. Rimsza, Rita M. Brazier, James R. Cook, Raymond R. Tubbs, Andreas Rosenwald, Jan Delabie, Randy D. Gascoyne **Collection and assembly of data:** Nathalie A. Johnson, Graham W. Slack, Kerry J. Savage, Joseph M. Connors, David W. Scott, King L. Tan, Christian Steidl, Laurie H. Sehn, Wing C. Chan, Javeed Iqbal, Paul N. Meyer, Georg Lenz, George Wright, Lisa M. Rimsza, Carlo Valentino, Patrick Brunhoeber, Thomas M. Grogan, Rita M. Brazier, James R. Cook, Raymond R. Tubbs, Dennis D. Weisenburger, Elias Campo, Andreas Rosenwald, German Ott, Jan Delabie, Elaine S. Jaffe, Louis M. Staudt, Randy D. Gascoyne **Data analysis and interpretation:** Nathalie A. Johnson, Susana Ben-Neriah, Sanja Rogic, Christian Steidl, Christina Holcroft **Manuscript writing:** All authors **Final approval of manuscript:** All authors

#### REFERENCES

1. Swerdlow SH, Campo E, Harris NL, et al: WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France, IARC Press, 2008, pp 265-266
2. Alizadeh AA, Eisen MB, Davis RE, et al: Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403:503-511, 2000
3. Lenz G, Wright G, Dave SS, et al: Stromal gene signatures in large-B-cell lymphomas. *N Engl J Med* 359:2313-2323, 2008
4. Choi WW, Weisenburger DD, Greiner TC, et al: A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. *Clin Cancer Res* 15:5494-5502, 2009
5. Hans CP, Weisenburger DD, Greiner TC, et al: Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood* 103:275-282, 2004
6. Meyer PN, Fu K, Greiner TC, et al: Immunohistochemical methods for predicting cell of origin and survival in patients with diffuse large B-cell lymphoma treated with rituximab. *J Clin Oncol* 29:200-207, 2011
7. Gutiérrez-García G, Cardesa-Salzmán T, Climent F, et al: Gene-expression profiling and not immunophenotypic algorithms predicts prognosis in patients with diffuse large B-cell lymphoma treated with immunochemotherapy. *Blood* 117:4836-4843, 2011
8. Lenz G, Staudt LM: Aggressive lymphomas. *N Engl J Med* 362:1417-1429, 2010
9. Rui L, Schmitz R, Ceribelli M, et al: Malignant pirates of the immune system. *Nat Immunol* 12:933-940, 2011
10. Adams JM, Harris AW, Pinkert CA, et al: The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* 318:533-538, 1985

11. Vaux DL, Cory S, Adams JM: Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* 335:440-442, 1988
12. Iqbal J, Neppalli VT, Wright G, et al: BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma. *J Clin Oncol* 24:961-968, 2006
13. Iqbal J, Sanger WG, Horsman DE, et al: BCL2 translocation defines a unique tumor subset within the germinal center B-cell-like diffuse large B-cell lymphoma. *Am J Pathol* 165:159-166, 2004
14. Savage KJ, Johnson NA, Ben-Neriah S, et al: MYC gene rearrangements are associated with a poor prognosis in diffuse large B-cell lymphoma patients treated with R-CHOP chemotherapy. *Blood* 114:3533-3537, 2009
15. Rimsza LM, Leblanc ML, Unger JM, et al: Gene expression predicts overall survival in paraffin-embedded tissues of diffuse large B-cell lymphoma treated with R-CHOP. *Blood* 112:3425-3433, 2008
16. Barrans S, Crouch S, Smith A, et al: Rearrangement of MYC is associated with poor prognosis in patients with diffuse large B-cell lymphoma treated in the era of rituximab. *J Clin Oncol* 28:3360-3365, 2010
17. Aukema SM, Siebert R, Schuurin E, et al: Double-hit B-cell lymphomas. *Blood* 117:2319-2331, 2011
18. Snuderl M, Kolman OK, Chen YB, et al: B-cell lymphomas with concurrent IGH-BCL2 and MYC rearrangements are aggressive neoplasms with clinical and pathologic features distinct from Burkitt lymphoma and diffuse large B-cell lymphoma. *Am J Surg Pathol* 34:327-340, 2010
19. Johnson NA, Savage KJ, Ludkovski O, et al: Lymphomas with concurrent BCL2 and MYC translocations: The critical factors associated with survival. *Blood* 114:2273-2279, 2009
20. Gurel B, Iwata T, Koh CM, et al: Nuclear MYC protein overexpression is an early alteration in human prostate carcinogenesis. *Mod Pathol* 21:1156-1167, 2008
21. Kluk MJ, Chapuy B, Sinha P, et al: Immunohistochemical detection of MYC-driven diffuse large B-cell lymphomas. *PLoS one* 7:e33813, 2012
22. Green TM, Nielsen O, de Stricker K, et al: High levels of nuclear MYC protein predict the presence of MYC rearrangement in diffuse large B-cell lymphoma. *Am J Surg Pathol* 36:612-619, 2012
23. Iqbal J, Meyer PN, Smith LM, et al: BCL2 predicts survival in germinal center B-cell-like diffuse large B-cell lymphoma treated with CHOP-like therapy and rituximab. *Clin Cancer Res* 17:7785-7795, 2011
24. Dave SS, Fu K, Wright GW, et al: Molecular diagnosis of Burkitt's lymphoma. *N Engl J Med* 354:2431-2442, 2006
25. Wright G, Tan B, Rosenwald A, et al: A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. *Proc Natl Acad Sci U S A* 100:9991-9996, 2003
26. Camp RL, Dolled-Filhart M, Rimm DL: X-Tile: A new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. *Clin Cancer Res* 10:7252-7259, 2004
27. Kerosuo L, Piltti K, Fox H, et al: Myc increases self-renewal in neural progenitor cells through Miz-1. *J Cell Sci* 121:3941-3950, 2008
28. Chang TC, Yu D, Lee YS, et al: Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet* 40:43-50, 2008
29. van Riggelen J, Yetil A, Felsher DW: MYC as a regulator of ribosome biogenesis and protein synthesis. *Nat Rev Cancer* 10:301-309, 2010
30. Hoffman B, Liebermann DA: Apoptotic signaling by c-MYC. *Oncogene* 27:6462-6472, 2008
31. Wierstra I, Alves J: The c-myc promoter: Still MysterY and challenge. *Adv Cancer Res* 99:113-333, 2008
32. Choi PS, van Riggelen J, Gentles AJ, et al: Lymphomas that recur after MYC suppression continue to exhibit oncogene addiction. *Proc Natl Acad Sci U S A* 108:17432-17437, 2011
33. Vafa O, Wade M, Kern S, et al: C-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: A mechanism for oncogene-induced genetic instability. *Mol Cell* 9:1031-1044, 2002
34. Tzankov A, Zlobec I, Went P, et al: Prognostic immunophenotypic biomarker studies in diffuse large B cell lymphoma with special emphasis on rational determination of cut-off scores. *Leuk Lymphoma* 51:199-212, 2010
35. Salles G, de Jong D, Xie W, et al: Prognostic significance of immunohistochemical biomarkers in diffuse large B-cell lymphoma: A study from the Lunenburg Lymphoma Biomarker Consortium. *Blood* 117:7070-7078, 2011
36. McShane LM, Altman DG, Sauerbrei W, et al: REporting recommendations for tumor MARKer prognostic studies (REMARK). *Nat Clin Pract Urol* 2:416-422, 2005
37. Klapper W, Kreuz M, Kohler CW, et al: Patient age at diagnosis is associated with the molecular characteristics of diffuse large B-cell lymphoma. *Blood* 119:1882-1887, 2012
38. Liu Y, Hernandez AM, Shibata D, et al: BCL2 translocation frequency rises with age in humans. *Proc Natl Acad Sci U S A* 91:8910-8914, 1994
39. Jardin F: Classification of diffuse large B-cell lymphoma by immunohistochemistry demonstrates that elderly patients are more common in the non-GC subgroup and younger patients in the GC subgroup (reply). *Haematologica* 97:e4, 2012
40. Thunberg U, Amini RM, Linderth J, et al: BCL2 expression in de novo diffuse large B-cell lymphoma partly reflects normal differences in age distribution. *Br J Haematol* 146:683-684, 2009
41. Mounier N, Briere J, Gisselbrecht C, et al: Rituximab plus CHOP (R-CHOP) overcomes bcl-2-associated resistance to chemotherapy in elderly patients with diffuse large B-cell lymphoma (DLBCL). *Blood* 101:4279-4284, 2003
42. Winter JN, Weller EA, Horning SJ, et al: Prognostic significance of Bcl-6 protein expression in DLBCL treated with CHOP or R-CHOP: A prospective correlative study. *Blood* 107:4207-4213, 2006
43. Schmitt CA, Lowe SW: Bcl-2 mediates chemoresistance in matched pairs of primary E(mu)-myc lymphomas in vivo. *Blood Cells Mol Dis* 27:206-216, 2001
44. Mead GM, Barrans SL, Qian W, et al: A prospective clinicopathologic study of dose-modified CODOX-M/IVAC in patients with sporadic Burkitt lymphoma defined using cytogenetic and immunophenotypic criteria (MRC/NCRI LY10 trial). *Blood* 112:2248-2260, 2008
45. Oltersdorf T, Elmore SW, Shoemaker AR, et al: An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 435:677-681, 2005
46. Tse C, Shoemaker AR, Adickes J, et al: ABT-263: A potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res* 68:3421-3428, 2008
47. Mason KD, Vandenberg CJ, Scott CL, et al: In vivo efficacy of the Bcl-2 antagonist ABT-737 against aggressive Myc-driven lymphomas. *Proc Natl Acad Sci U S A* 105:17961-17966, 2008

### Affiliations

Nathalie A. Johnson and Christina Holcroft, Jewish General Hospital, Montreal, Quebec; Graham W. Slack, Kerry J. Savage, Joseph M. Connors, Susana Ben-Neriah, Sanja Rogic, David W. Scott, King L. Tan, Christian Steidl, Laurie H. Sehn, and Randy D. Gascoyne, British Columbia Cancer Agency, Vancouver, British Columbia, Canada; Wing C. Chan, Javeed Iqbal, Paul N. Meyer, and Dennis D. Weisenburger, University of Nebraska Medical Center, Omaha, NE; Georg Lenz, Oncology and Tumorimmunology, Charité-Universitätsmedizin, Berlin; Andreas Rosenwald and German Ott, University of Würzburg, Würzburg; German Ott, Robert-Bosch-Krankenhaus and Dr Margarete Fischer-Bosch Institute for Clinical Pharmacology, Stuttgart, Germany; George Wright, Elaine S. Jaffe, and Louis M. Staudt, National Cancer Institute, National Institutes of Health, Bethesda, MD; Lisa M. Rimsza, Carlo Valentino, Patrick Brunhoeber, and Thomas M. Grogan, University of Arizona, Tucson, AZ; Rita M. Braziel, Oregon Health and Science University, Portland, OR; James R. Cook and Raymond R. Tubbs, Cleveland Clinic, Cleveland, OH; Elias Campo, Hospital Clinic, Barcelona, Spain; and Jan Delabie, Radiumhospitalet, Oslo, Norway.