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Date:
2021-04-01

Citation:
Punnoose, E., Peale, F. V., Szafer-Glusman, E., Lei, G., Bourgon, R., Do, A. D., Kim, E., Zhang, L., Farinha, P., Gascoyne, R. D., Munoz, F. J., Martelli, M., Mottok, A., Salles, G. A., Sehn, L. H., Seymour, J. F., Trněný, M., Oestergaard, M. Z., Mundt, K. E. & Vitolo, U. (2021). BCL2 Expression in First-Line Diffuse Large B-Cell Lymphoma Identifies a Patient Population With Poor Prognosis. Clinical Lymphoma Myeloma and Leukemia, 21 (4), pp.267-278.e10. https://doi.org/10.1016/j.clml.2020.11.004.

Persistent Link:
https://hdl.handle.net/11343/273776

BCL2 Expression in First-Line Diffuse Large B-Cell Lymphoma Identifies a Patient Population With Poor Prognosis

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Abstract

The prognostic significance of B-cell lymphoma 2 (*BCL2*) expression in patients with diffuse large B-cell lymphoma (DLBCL) is conflicting. We developed an immunohistochemistry (IHC) algorithm and assay to determine *BCL2* expression and assessed the prognostic value of *BCL2* in newly diagnosed DLBCL cohorts. *BCL2* expression was associated with poorer progression-free survival. Findings support use of our *BCL2* IHC scoring system and assay to select *BCL2*-positive patients for future studies.

Introduction: The prognostic value of B-cell lymphoma 2 (*BCL2*) expression in de novo diffuse large B-cell lymphoma (DLBCL) treated with immunochemotherapy is of interest to define a target patient population for clinical development of *BCL2* inhibitors. We aimed to develop a reproducible immunohistochemistry algorithm and assay to determine *BCL2* protein expression and assess the prognostic value of *BCL2* in newly diagnosed DLBCL cohorts. **Patients and Methods:** The prospectively defined algorithm incorporated *BCL2* staining intensity and percentage of *BCL2*-positive cells. Functionally relevant cutoffs were based on the sensitivity of lymphoma cell lines to venetoclax. This assay was highly reproducible across laboratories. The prognostic impact of *BCL2* expression was assessed in DLBCL patients from the phase 3 MAIN (n = 230) and GOYA (n = 366) trials, and a population-based registry (n = 310). **Results:** Approximately 50% of tumors were *BCL2* positive, with a higher frequency in high International Prognostic Index (IPI) and activated B-cell–like DLBCL subgroups. *BCL2* expression was associated with poorer progression-free survival in the MAIN study (hazard ratio [HR], 1.66; 95% confidence interval [CI], 0.81-3.40; multivariate Cox regression adjusted for IPI and cell of origin). This trend was confirmed in the GOYA and registry cohorts in adjusted multivariate analyses (GOYA: HR, 1.72; 95% CI, 1.05-2.82; registry: HR, 1.89; 95% CI, 1.29-2.78). Patients with *BCL2* immunohistochemistry-positive and IPI-high disease had the poorest prognosis: 3-year progression-free survival rates were 51% (GOYA) and 37% (registry). **Conclusion:** Findings support use of our *BCL2* immunohistochemistry scoring system and assay to select patients with *BCL2*-positive tumors for future studies.

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Submitted: Aug 11, 2020; Revised: Oct 27, 2020; Accepted: Nov 5, 2020; Epub: Nov 10, 2020

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https://doi.org/10.1016/j.clml.2020.11.004

Clinical Lymphoma, Myeloma & Leukemia, Vol. 21, No. 4, 267-78
Keywords: ABC DLBCL subtype, Biomarkers, Cell of origin, GCB DLBCL subtype, Immunohistochemistry

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common histologic subtype of non-Hodgkin lymphoma (NHL), accounting for approximately 30% of cases.[1-3] While more than half of patients with DLBCL are cured with the current standard regimen of rituximab (R) plus cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy, a significant number of patients experience disease relapse.[4] Outcomes for patients with DLBCL refractory to, or relapsing after, first-line therapy are poor[5]; thus, markers are needed to identify patients with poor prognosis, especially those who may benefit from novel agents in development.

The International Prognostic Index (IPI) is a clinical prognostic scoring system, developed in 1993 and validated in the rituximab era, which incorporates independent prognostic factors to predict the risk of disease recurrence.[6,7] Within IPI-defined groups, however, molecular characteristics of the disease independently contribute to outcome.[8,9] For example, activated B-cell–like (ABC) and germinal center B-cell–like (GCB) cell-of-origin (COO) DLBCL subtypes, identified by gene-expression profiling, are each associated with a distinct prognosis.[10,11] Novel therapies that target subtype-specific pathways, including Bruton's tyrosine kinase inhibitors, proteasome inhibitors, and immunomodulatory agents, have been under evaluation for ABC (or non-GCB) DLBCL.[12-15]

Beyond the impact of COO, expression of biologically relevant proteins contributes to the prognostic value in DLBCL. In particular, strong expression of B-cell lymphoma 2 (*BCL2*), an antiapoptotic regulator linked to tumor aggressiveness and resistance to cytotoxic treatment, has been associated with adverse prognosis (Supplementary Table 1 in the online version).[16,17] However, findings on the prognostic impact of *BCL2* expression after the introduction of R to the CHOP regimen,[18,19] and within the context of COO molecular subtypes[20] and IPI status,[19] have been conflicting. While the immunohistochemistry (IHC) antibody (clone 124) that is used to define *BCL2* expression has remained largely consistent across studies, the IHC cutoffs used to define *BCL2* as a prognostic biomarker have varied greatly, ranging from > 1% to > 75% of positively stained tumor cells.[17,19,21] This may partially explain the observed differences in the prognostic impact of *BCL2*.[22,23] Moreover, the intensity of *BCL2* staining has not historically been considered in these analyses, with the exception of a recent study using an algorithm described by Tsuyama et al,[23] which highlighted the utility of incorporating both the proportion of positive cells and the intensity of staining for *BCL2*.

After development of the highly selective *BCL2* inhibitor venetoclax as a treatment for hematologic malignancies,[22-24] a refined *BCL2* scoring algorithm and assay that employs a functionally relevant cutoff based on venetoclax sensitivity is desirable. Validated analytical techniques, for example with high assay precision, are prerequisites for use of such an assay in routine clinical testing, and in particular for patient selection for therapy. Our objectives for the current analyses were 2-fold: (1) to develop a simple, reproducible scoring system and assay for categorizing *BCL2* expression by IHC, incorporating a biologically relevant cutoff for *BCL2* staining; (2) to utilize this scoring system to assess the prognostic impact of *BCL2* in patients with DLBCL who received first-line CHOP plus an anti-CD20 antibody in the phase 3 MAIN[25] and GOYA[26] studies, and in a real-world patient registry data set from British Columbia Cancer (BC Cancer).[27]

Patients and Methods

Patient Cohorts and Study Conduct

Detailed methods for the randomized, placebo-controlled, phase 3 MAIN study (ClinicalTrials.gov NCT00486759) have been described previously.[25] Briefly, adult patients aged ≥ 18 years with newly diagnosed CD20-positive DLBCL were randomized to R-CHOP or R-CHOP plus bevacizumab. MAIN was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice.[25] The protocol and all accompanying materials that were provided to patients were reviewed and approved by institutional ethics committees or institutional review boards. Informed consent was obtained from all patients contributing tumor specimens for biomarker analysis at the time of data cutoff (November 30, 2011). The phase 3 GOYA trial (data cutoff April 30, 2016) randomized adult patients with previously untreated CD20-positive DLBCL to R-CHOP or obinutuzumab (G)-CHOP.[26] GOYA (NCT01287741) was conducted in accordance with the European Clinical Trial Directive for European centers and Good Clinical Practice. The protocol was approved by the ethics committees of participating centers. Written informed consent was obtained from all patients. The third patient cohort investigated in this study comprised patients who received R-CHOP in a population-based DLBCL registry (BC Cancer) that has been described previously.[27] The BC Cancer registry study received approval from the University of British Columbia–British Columbia Cancer Research Ethics Board.

In Vitro Cell-Growth Inhibition

Cells from 26 NHL cell lines (described in Supplementary Table 2 in the online version) were plated at 1500 to 5000 cells per well in 25 µL medium in 384-well plates. Medium (25 µL) containing venetoclax was added at 9 concentrations in 3-fold serial dilutions with a maximum dose of 5 µM. Cells were incubated in the presence of drug for 3 days and viability was measured using CellTiterGlo (Promega, Madison, W). Half-maximal inhibitory concentration (IC_{50}) values were calculated with a 4-parameter fit using XLfit (IDBS, Boston, MA) over an average of at least 3 independent assays. The sensitivity cutoff was defined as 1 µM, the value used across in vitro studies to assess sensitivity to venetoclax,[28] which corresponds to efficacious doses in xenograft studies (100 mg/kg)[28,29] and to doses achievable in the clinical setting (400 mg).

Immunohistochemistry

Scoring Algorithm. *BCL2* expression levels in formalin-fixed, paraffin-embedded (FFPE) pellets from NHL cell lines were investigated. The scoring algorithm was developed to incorporate both the percentage of positively stained tumor cells (≥ 50% of tumor cells as previously defined[27,30]) and the intensity of tumor cell staining. Mantle zone B cells and paracortical T cells in normal tonsils were used as references for "moderate" *BCL2* IHC staining intensity. Tumor cells with a signal weaker or stronger than this were assigned an intensity of "weak" or "strong", respectively. On the basis of ≥ 50% of tumor cells being assigned to 1 of 4 groups—no expression, weak, moderate, or strong expression—the algorithm defines 1 of 4 possible IHC scores: 0, 1+, 2+, or 3+. Samples were designated *BCL2* negative if the tumor scored 0 or 1+, and *BCL2* positive if the tumor scored 2+ or 3+. Samples were considered *MYC* (myelocytomatosis) positive if ≥ 40% of tumor cells[27,30] showed *MYC* nuclear staining at any intensity above background. Tumors that were *BCL2* positive and *MYC* positive by IHC were classified as double-expressing (DE) lymphoma.

Application of Scoring Algorithm to Patient Samples. The same *BCL2* and *MYC* scoring algorithms were used for all samples in the studies reported here. The cell lines and MAIN cohort were run first using an earlier research-use-only version of the *BCL2* and *MYC* assays, and the GOYA study and DLBCL registry cohorts were subsequently run using the Ventana investigational assay (Ventana Medical Systems, Inc, Tucson, AZ, United States; Supplementary Table 3 in the online version), as described below. FFPE tumor specimens from MAIN were arranged on 8 tissue microarrays (TMAs), constructed at 5 centers. *BCL2* IHC was assessed using the anti-*BCL2* (clone 124) mouse monoclonal antibody that recognizes an epitope of *BCL2* spanning amino acids 41-54.[31] As some reports questioned the sensitivity of clone 124 to detect *BCL2* protein expression,[32] consecutive sections of the same TMA were additionally assessed using other *BCL2* antibodies (which were not as widely known, therefore their relative performance had not yet been described): clone E17 (epitope 61-76 amino acids[33]) and SP66 rabbit monoclonal antibodies (Ventana Medical Systems Inc[32]). *MYC* IHC was assessed using the clone Y69 Epitomics antibody.[34] Subsequently, TMAs from the registry cohort were processed with the Ventana *BCL2* (clone 124) and *MYC* (Y69) investigational assays, which use the same antibody clones as in MAIN but are run on the Ventana BenchMark ULTRA fully automated system. TMAs from the registry cohort were assessed by pathologists blinded to previous IHC assessments in this cohort. The Ventana *BCL2* (124) and Ventana *MYC* (Y69) investigational assays have not been approved by the US Food and Drug Administration and are not intended for clinical diagnostic use.

Reproducibility Assessment for the Ventana BCL2 *Investigational Assay.* Reproducibility studies were carried out to assess the performance of the Ventana *BCL2* investigational assay and its potential utility for patient selection in investigational studies. The reproducibility studies were performed on a separate set of samples, other than the clinical cohorts. These were commercially acquired DLBCL samples, verified by the Ventana pathologists and with sufficient tumor tissue to support the needs of the interreader and intrareader reproducibility studies. Reader precision was assessed by 3 pathologists independently scoring slides with tissue sections stained for *BCL2* in 104 DLBCL cases. The slides were then rescored after a 2-week period for comparison of results. For interlaboratory reproducibility assessment, 28 DLBCL tissue samples representing the full range of IHC assay outcomes were sent to 3 laboratories and independently stained and scored over 5 nonconsecutive days within a minimum period of 20 days. The study included 1 or more instruments per site and was scored by 2 pathologists per site. Reproducibility assessment was carried out as part of the development of the Ventana *BCL2* investigational assay.

Using the proposed scoring algorithm, the concordance of different antibody clones on *BCL2* status was assessed in replicate TMAs from 180 MAIN study patients, with staining conditions adjusted to result in similar staining intensities in control tonsil sections. Concordance between the Ventana *BCL2* investigational assay and *BCL2* status by local testing was also assessed in the BC Cancer registry cohort (n = 310).

Messenger RNA (mRNA) Analysis

RNA was extracted from FFPE tissues from patients in the GOYA study using the RNeasy FFPE kit (Qiagen, Venlo, Netherlands) and gene expression was analyzed by RNA sequencing (RNA-Seq). Analysis of RNA-Seq data was performed in the R statistical computing language. Raw reads were summarized as per gene counts, and were normalized using the robust library size estimate from DESeq2.[35]

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) was performed in a central laboratory (HistoGeneX, Antwerp, Belgium) on diagnostic FFPE tissue sections from patients in the GOYA study. Vysis LSI Dual Color Break Apart FISH probes were used to identify *BCL2* and *MYC* translocations, as previously described,[36] with FISH positivity defined as ≥ 50% of nuclei with break-apart signal. Fifty percent cutoff definition is based on assay validation of the laboratory-developed test including accuracy and precision in normal tonsils to define a negative cutoff value and in tissue blocks of follicular lymphoma and DLBCL with known translocation status used as positive controls.

COO Subtyping by Gene Expression Signature

DLBCL COO subtypes from FFPE tumor tissue were determined using a quantitative real-time polymerase chain reaction assay on a Fluidigm high-throughput platform[37]; the linear-predictor score classifier[38] was adapted to the Fluidigm assay (referred to as Fluidigm COO Classifier assay; Supplementary Materials and Methods in the online version). This custom COO classifier was designed to analyze samples before Nanostring Lymph2Cx was available. COO subtypes were also assigned using the Lymph2Cx assay based on the Nanostring gene expression profiling platform and COO classification of the GOYA and registry cohorts was performed by Nanostring assay, as described previously.[39] For validation of the COO assay and its performance in relation to the Nanostring assay in the MAIN cohort (n = 160); see Supplementary Results and Supplementary Figure 1 in the online version.

Statistical Methods

Patients from both arms of the MAIN and GOYA studies were pooled for analysis, as the investigational arms in each study (R-CHOP plus bevacizumab and G-CHOP, respectively) had showed no difference in efficacy in the overall population, and exploratory analyses of the effect of investigational treatment in biologic subsets showed no significant differential impact. Association of baseline *BCL2* expression (*BCL2* positive vs. *BCL2* negative) with the clinical endpoint of progression-free survival (PFS) was assessed using Cox regression analysis. Covariates for Cox regression analysis were IPI score (low [0-2] or high [3-5]), treatment (MAIN: with or without bevacizumab; GOYA: R-CHOP or G-CHOP), and planned number of CHOP chemotherapy cycles (6 or 8), representing the treatment arms and stratification factors used in the studies, and COO subtype (GCB or ABC). Associations between *BCL2* IHC positivity or negativity, against COO or IPI were tested with Fisher's exact test. There were no preplanned sample size or power calculations for this retrospective exploratory biomarker analysis. Analysis was performed by all authors and all authors had access to primary clinical trial data.

Data-Sharing Statement

Qualified researchers may request access to individual patient-level data through the clinical study data request platform (www.clinicalstudydatarequest.com). Further details on F. Hoffmann-La Roche Ltd criteria for eligible studies are available at https://clinicalstudydatarequest.com/Study-Sponsors/Study-Sponsors-Roche.aspx. For further details on F. Hoffmann-La Roche Ltd Global Policy on the Sharing of Clinical Information and how to request access to related clinical study documents, visit https://www.roche.com/research_and_development/who_we_are_how_we_work/clinical_trials/our_commitment_to_data_sharing.htm.

Results

Patient Populations

Tissue specimens for *BCL2* IHC analysis were available for 184 (23%) of 787 patients from MAIN, 366 (26%) of 1418 patients from GOYA, and 310 patients from the BC Cancer registry (*BCL2* biomarker-evaluable populations). To ensure that *BCL2* biomarker-evaluable populations were representative of the overall intention-to-treat (ITT) populations, baseline demographics and characteristics for these patient groups in the MAIN and GOYA trials were compared and shown to be similar, with some exceptions, such as race (fewer Asian patients in the biomarker population in both studies) and bulky disease (fewer patients with disease $\geq$ 7.5 cm in the MAIN biomarker population; Supplementary Table 4 in the online version). There were no major differences between the patient characteristics of the trial populations and those previously reported for the registry cohort,[27] except that the latter group included a slightly higher proportion of males and patients with a higher Eastern Cooperative Oncology Group performance status.

For the MAIN and GOYA studies, PFS plots were overlapping between the *BCL2* biomarker-evaluable and overall ITT populations (Supplementary Figure 2A in the online version), and the expected association between IPI score and prognosis was observed among the *BCL2* biomarker-evaluable populations (MAIN: hazard ratio [HR], 3.5; 95% confidence interval [CI], 1.98-6.19; $P < .0001$; GOYA: HR, 2.16; 95% CI, 1.46-3.2; $P = .0001$; Supplementary Figure 2B in the online version). As expected, a trend toward inferior outcome for ABC versus GCB DLBCL was also seen in the *BCL2* biomarker-evaluable population (MAIN: HR, 1.80; 95% CI, 0.96-3.39; $P = .069$; GOYA: HR, 1.95; 95% CI, 1.22-3.12; $P = .0054$; Supplementary Figure 2C in the online version). These data indicate that the biomarker-evaluable population is representative of the overall ITT population.

Development of BCL2 *IHC Assay*

Of 13 NHL cell lines with *BCL2* expression greater than or equal to an IHC score of 2+, 10 (78%) cell lines showed high sensitivity ($IC_{50} < 1$ μM) to single-agent venetoclax (Figure 1A). Conversely, all 13 cell lines with *BCL2* expression below an IHC score of 2+ showed lower sensitivity to venetoclax ($IC_{50} \geq 1$ μM). On the basis of these findings, a *BCL2* IHC score of 2+ was used to define the cutoff for *BCL2* positivity in the tumor-cell population (Figure 1B). The concordance of *BCL2* status by different antibody clones in replicate TMAs from MAIN study patients is shown in Figure 2A. Analysis of *BCL2* status showed a substantially similar level of *BCL2* protein expression and > 95% concordance among the clone 124, E17, and SP66 antibodies (Figure 2B). The frequency of *BCL2*-positive tumor samples was 48.3% (87/180) for clone 124, 50.6% (91/180) for E17, and 46.7% (84/180) for SP66. Overall, clone 124 detected > 95% of the *BCL2*-positive cases detected by any of the *BCL2* antibodies tested in this DLBCL sample set; these data provided supportive evidence for the choice of clone 124 for development of the investigational assay.

Reproducibility of Ventana BCL2 *Investigational Assay*

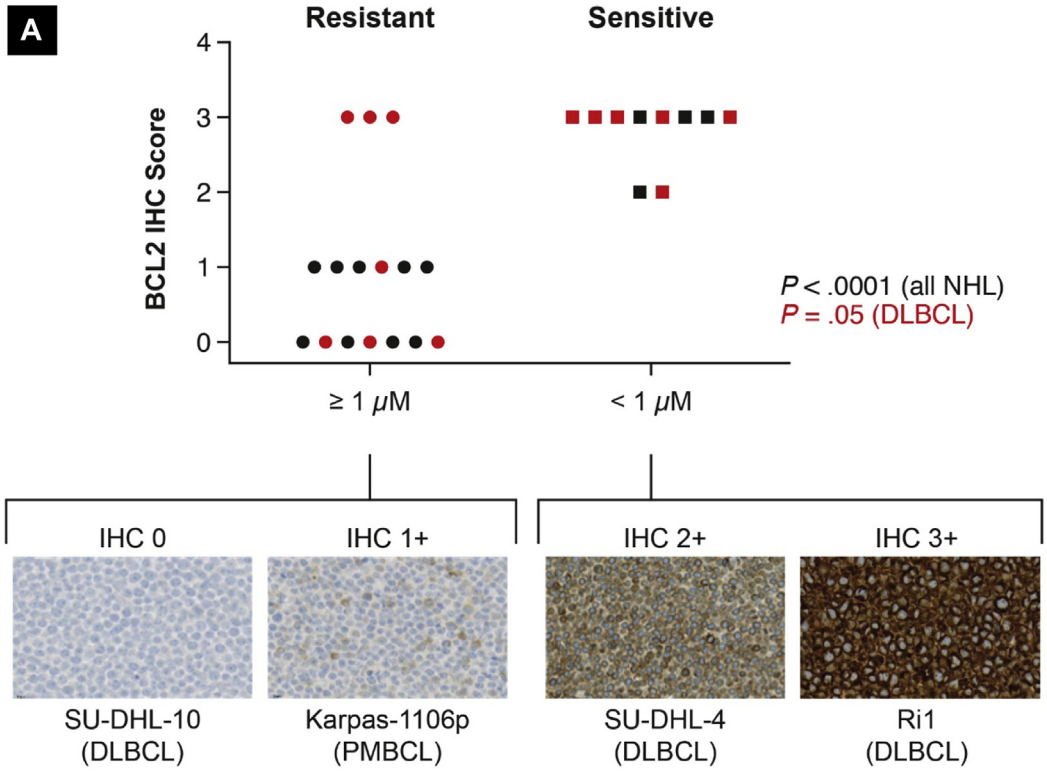
Reader concordance for the Ventana *BCL2* investigational assay was $\geq$ 90% for both interobserver and intraobserver agreement as well as interlaboratory reproducibility across 3 trained, but independent, clinical laboratories.

Investigation of concordance between the Ventana assay and *BCL2* status assessed by local testing in the BC Cancer registry cohort (n = 310) revealed a high concordance overall (87%) and in *BCL2*-negative patients (97%). Of patients identified as *BCL2* positive in the BC Cancer cohort, 15% were reported as *BCL2* negative by the Ventana investigational assay, with discordance primarily occurring in patients with weak *BCL2* staining intensity (IHC 1+). When exploring how the Ventana *BCL2* investigational assay compared with orthogonal assessments of *BCL2*, *BCL2* IHC status was found to be strongly correlated with *BCL2* mRNA expression assessed by RNA-Seq (n = 433; $P < .001$ by 2-sample *t* test) (Figure 2C) and with the presence of *BCL2* translocations assessed by FISH in patient samples from the GOYA study (Figure 2D).

BCL2 *Expression and Its Association With Clinical and Molecular Subgroups*

In MAIN, the frequency of each of the 4 *BCL2* IHC staining scores was approximately equal: 24%, 28%, 20%, and 28% at 0, 1+, 2+, 3+, respectively, resulting in a 48% rate of *BCL2*-

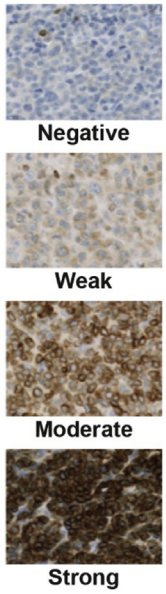
Figure 1 ***BCL2*** **Expression in NHL Cell Lines. (A) NHL Cell Lines Sensitive to Venetoclax Are High in** ***BCL2*** **Expression.** ***BCL2*** **IHC Scores in NHL Cell Lines Categorized Into "Sensitive" and "Resistant" Groups Based on IC_{50} at 1 μM. DLBCL Cell Lines Are Shown in Red. IHC Images From Representative "Sensitive" and "Resistant" Cell Lines. (B)** ***BCL2*** **IHC Scoring Algorithm. (Right) Representative Images of Negative to Strong** ***BCL2*** **Expression in DLBCL Cases. (Left) Algorithm Results in 1 of 4 Possible IHC Clinical SCORES: 0, 1+, 2+, or 3+. Clinical Status Is** ***BCL2*** **Negative If Tumor Has a Clinical Score of 0 or 1+, and** ***BCL2*** **Positive With a Clinical Score of 2+ or 3+**



B

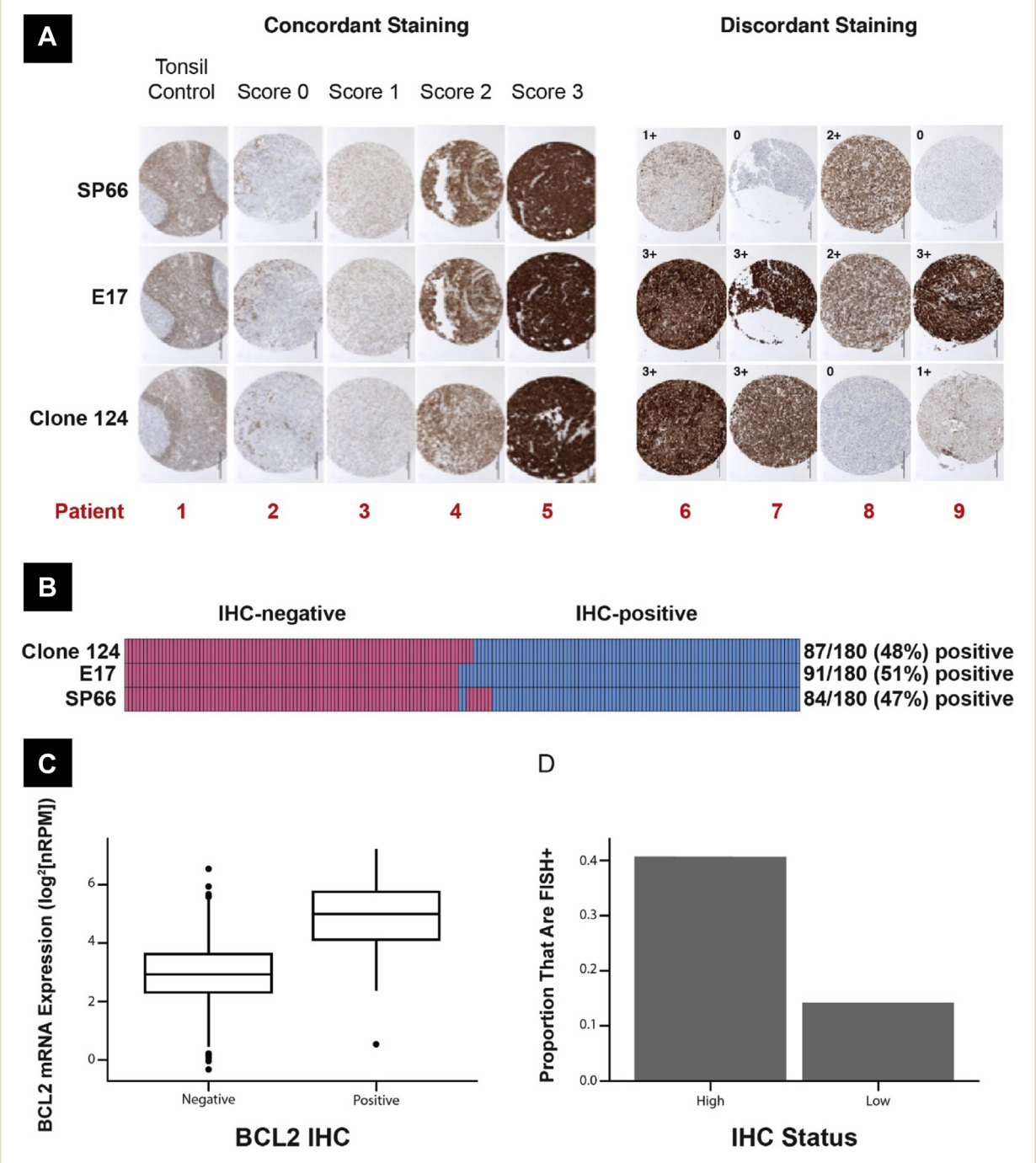
Clinical Status	Description	Clinical Score
Negative	No staining in tumor cells or < 50% of tumor cells with cytoplasmic BCL2 staining of any intensity	0
	≥ 50% of tumor cells with WEAK or higher cytoplasmatic BCL2 staining, but < 50% of tumor cells with MODERATE or STRONG staining intensity	1+
Positive	≥ 50% of tumor cells with MODERATE or higher cytoplasmatic BCL2 staining, but < 50% of tumor cells with STRONG staining intensity	2+
	≥ 50% of tumor cells with STRONG cytoplasmatic BCL2 staining	3+

- Mantle zone B cells and paracortical lymphocytes from a normal tonsil are used as reference for "moderate" BCL2 IHC staining
- Staining intensity for "BCL2-positive" is defined by threshold of expression associated with venetoclax drug sensitivity in lymphoma cell lines



Abbreviations: *BCL2* = B-cell lymphoma 2; DLBCL = diffuse large B-cell lymphoma; IC_{50} = half-maximal inhibitory concentration; IHC = immunohistochemistry; NHL = non-Hodgkin lymphoma; PMBCL = primary mediastinal large B-cell lymphoma.

Figure 2 Characterization of *BCL2* IHC Assay. (A) Representative Examples of *BCL2* Staining Using Clones 124, E17, and SP66. (B) Concordant *BCL2* Classification Based on IHC Staining Using 3 Antibodies. (C) *BCL2* IHC Status Is Associated With *BCL2* mRNA Expression (n = 433; $P < .001$ by 2-Sample *t* Test) in Samples From GOYA Study. (D) *BCL2* IHC Status Is Associated With *BCL2* Translocations (n = 455; OR, 4.15 [95% CI, 2.57-6.85], $P < .001$ by Fisher Exact Test) in Samples From GOYA Study



Abbreviations: *BCL2* = B-cell lymphoma 2; CI = confidence interval; FISH = fluorescence in situ hybridization; IHC = immunohistochemistry; mRNA = messenger RNA; nRPM = normalized reads per million; OR = odds ratio.

positivity (Table 1). In the GOYA trial and registry cohorts, 49% and 58% of evaluable patients were *BCL2* positive, respectively (Table 1).

BCL2-positive tumor samples were more frequent in the high-IPI, poor prognostic group than in the low-IPI, favorable prognostic group in both the MAIN and GOYA studies, but were

Table 1 Prevalence of *BCL2* IHC Positivity in 3 Patient Cohorts, in All Patients, and According to COO Subtype and IPI Score

Cohort	MAIN		GOYA		BC Cancer Registry	
	BCL2 IHC Positive, N/Tested (%)	OR (95% CI) *P*	*BCL2* IHC Positive, N/Tested (%)	OR (95% CI) *P*	*BCL2* IHC Positive, N/Tested (%)	OR (95% CI) *P*
All patients	88/184 (48)		178/366 (49)		180/310 (58)	
COO subtype		0.38		0.57		0.41
GCB	26/65 (40)	(0.16-0.90)	119/191 (62)	(0.32-0.99)	86/165 (52)	(0.23-0.71)
ABC	28/44 (64)	$P = .02$	76/102 (75)	$P = .04$	75/103 (73)	$P < .001$
IPI score		0.50		0.62		1.03
Low (0-2)	43/106 (41)	(0.27-0.95)	87/201 (43)	(0.40-0.96)	107/185 (58)	(0.61-1.74)
High (3-5)	45/78 (58)	$P = .03$	91/165 (55)	$P = .03$	56/98 (57)	$P = 1$

Associations between *BCL2* IHC$^{+/-}$ against COO or IPI were tested by Fisher's exact test.
Abbreviations: ABC = activated B-cell–like; BC = British Columbia; BCLC2 = B-cell lymphoma 2; CI = confidence interval; COO = cell of origin; GCB = germinal center B-cell–like; IHC = immunohistochemistry; IPI = International Prognostic Index; OR = odds ratio.

detected in similar proportions in both IPI prognostic groups in the registry cohort (Table 1). *BCL2*-positive samples were also more frequent in the ABC subtype than in the GCB subtype in all 3 patient cohorts (difference in frequency: MAIN: 24%; 95% CI, 5-40; GOYA: 13%; 95% CI, 2-24; registry: 21%; 95% CI, 10-32; Table 1).

Association of BCL2 *Protein Expression With Clinical Prognosis*

Patients with *BCL2*-positive disease showed a trend toward poorer PFS compared with those with *BCL2*-negative disease across the GOYA and BC Cancer registry cohorts, and a similar trend was observed in the MAIN cohort using multivariate analysis (Table 2; Figure 3). Association in COO and IPI subgroups was similar to overall trend: *BCL2* positivity was associated with a trend toward inferior outcome in both GCB DLBCL (GOYA: HR, 1.86; 95% CI, 1.00-3.45; BC Cancer registry: HR, 1.85; 95% CI, 1.08-3.19) and ABC DLBCL (GOYA: HR, 1.48; 95% CI, 0.67-3.24; BC Cancer registry: HR, 1.55; 95% CI, 0.89-2.71), but differences were small and not statistically significant (Figure 4A-D). Similarly, a trend for *BCL2* positivity and association with inferior PFS was observed in both high- and low-IPI groups (Figure 4E, F) in the GOYA and BC Cancer registry cohorts. Patients with *BCL2*-positive high-IPI disease were identified as the subgroup with the worst prognosis, with 3-year PFS rates of 51% (95% CI, 39-63) in GOYA and 37% (95% CI, 25-50) in the BC Cancer registry.

BCL2 positivity was associated with a trend toward inferior PFS regardless of *MYC* expression in the BC Cancer registry cohort (Supplementary Figure 3 in the online version). Patients with DE disease (those with dual expression of *BCL2* and *MYC*) had inferior outcomes relative to those without DE disease (HR, 1.93; 95% CI, 1.34-2.78), with 3-year PFS rates of 55% in patients with DE disease. The high risk observed here in patients with DE disease identified using the Ventana *BCL2* and *MYC* assays is similar to the inferior PFS for patients with DE disease versus patients without DE disease in matched patient samples using the BC Cancer registry pathology laboratory assays (HR, 2.25; 95% CI, 1.51-3.35; 3-year PFS rate of 50%).

In addition, overall survival multivariate analysis was available from the GOYA cohort. *BCL2* positivity was associated with a trend toward inferior overall survival (HR, 1.31; 95% CI, 0.80-2.13), consistent with the trend observed in PFS (Supplementary Figure 4 in the online version).

Discussion

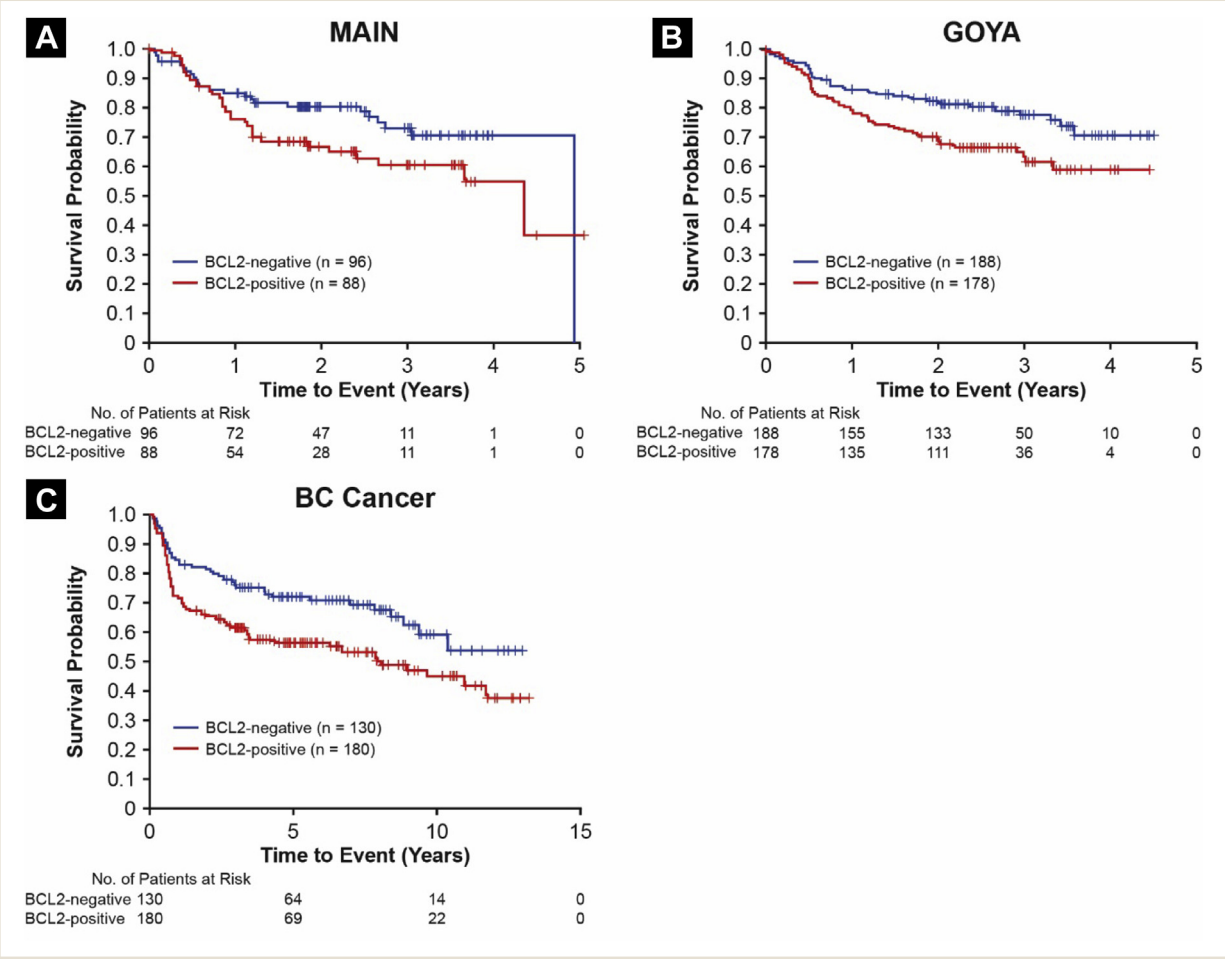
In the current study, we have developed and assessed a robust *BCL2* IHC scoring algorithm and threshold of expression that are consistent with sensitivity of NHL cell lines to *BCL2* inhibition. This scoring algorithm takes into account the dynamic range of *BCL2* expression in IHC-stained tumor tissue (incorporates the intensity of tumor cell staining in addition to the percentage of positively stained tumor cells), thereby building on previous IHC

Table 2 Prognostic Impact of *BCL2* IHC Positivity in 3 Patient Cohorts

Cohort (N)	IHC Status, N (%)	3-year PFS, % (95% CI)	Multivariate HR (95% CI)	Multivariate Model
MAIN (184)	*BCL2* positive: 88 (48) *BCL2* negative: 96 (52)	61 (48-71) 71 (58-80)	1.66 (0.81-3.40)	Adjusted for treatment, IPI, and COO
GOYA (366)	*BCL2* positive: 178 (49) *BCL2* negative: 188 (51)	63 (55-71) 78 (70-84)	1.72 (1.05-2.82)	Adjusted for treatment, IPI, and COO
BC Cancer registry (310)	*BCL2* positive: 180 (58) *BCL2* negative: 130 (42)	62 (54-68) 76 (67-82)	1.89 (1.29-2.78)	Adjusted for IPI and COO

Abbreviations: BCLC2 = B-cell lymphoma 2; CI = confidence interval; COO = cell of origin; HR = hazard ratio; IHC = immunohistochemistry; IPI = International Prognostic Index; PFS = progression-free survival.

Figure 3 Kaplan-Meier Curves for PFS for *BCL2*-Positive Versus *BCL2*-Negative Expression in *BCL2* IHC Populations in MAIN (A), GOYA (B), and BC Cancer Registry (C) Cohorts



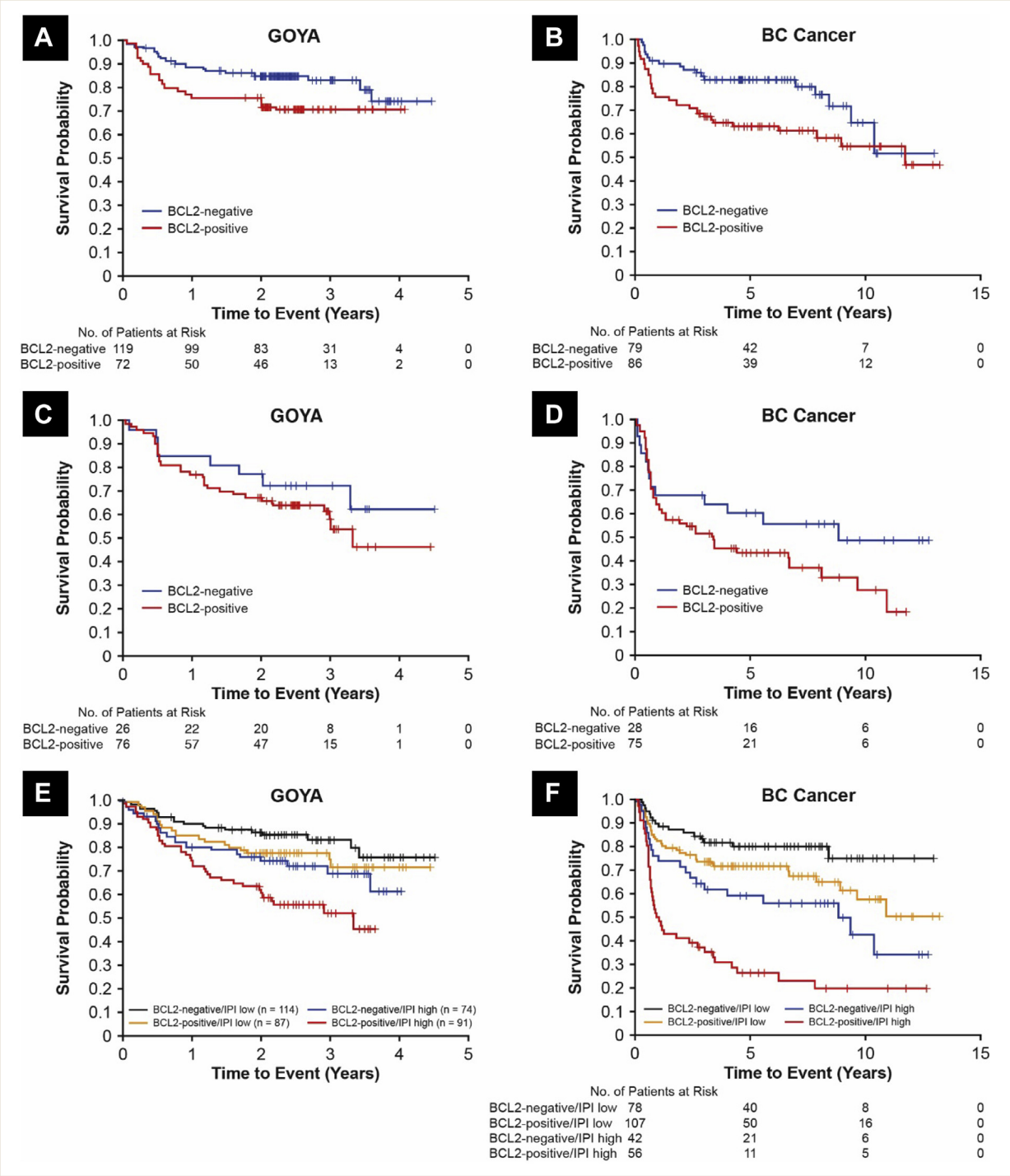
Abbreviations: BC = British Columbia; *BCL2* = B-cell lymphoma 2; PFS = progression-free survival.

cutoff definitions based only on ≥ 50% of tumor cells stained positive,[27,30] for which evidence of prognostic value already existed (and which is recommended by updated World Health Organization criteria[40]). Here, we define a "positive stain" based on staining intensity levels, while requiring at least 50% of tumor cells to exceed a defined staining level. In contrast to previous observation,[32] *BCL2* IHC status determined by our scoring algorithm was reproducible across the 3 different *BCL2* antibodies tested (E17, 124, and SP66), using tonsil as control for staining intensity. This demonstrates the robustness of assessing *BCL2* expression by IHC. This new algorithm could have utility for selection of patients with adverse risk by *BCL2* status, and may also have value in the investigational evaluation of *BCL2* inhibitors such as venetoclax in DLBCL.

By incorporating both staining intensity/percentage of tumor cell positivity and standardized staining techniques, the Ventana *BCL2* investigational assay demonstrated ≥ 90% agreement among the pathologists' scores and between laboratories. Notably, this is an improvement over the reproducibility for *BCL2* by IHC (47% agreement) reported by the Lunenburg Lymphoma Biomarker Consortium in their assessment of potential prognostic markers in DLBCL.[22] Consistent with our approach, they observed an improvement in reproducibility by specifying staining intensity for *BCL2*. In addition, the Ventana *BCL2* investigational assay incorporates recommendations from the Lunenburg Lymphoma Biomarker Consortium study, including standardization of staining techniques, a simplified scoring system with only 2 categories, and removal of biologically uninformative cutoff points. To the latter point, our scoring algorithm has a simple requirement for 50% tumor cell positivity and a biologically relevant cutoff for staining intensity, based on sensitivity of cell lines to venetoclax, which results in a dichotomized category of *BCL2*-positive or *BCL2*-negative status.

We observed high concordance between the Ventana *BCL2* investigational assay and *BCL2* status determined by the BC Cancer registry pathology laboratory, noting that these methods both use the clone 124 antibody and a 50% tumor cell positivity cutoff. Supporting these findings, it was reported recently in a large lymphoma cohort that *BCL2* IHC is highly reproducible when cutoff

Figure 4 Kaplan-Meier Curves for PFS in *BCL2*-Positive Versus *BCL2*-Negative Patients. GCB Molecular Subtypes of GOYA (A) and BC Cancer Registry (B) Cohorts. ABC Molecular Subtypes of GOYA (C) and BC Cancer Registry (D) Cohorts. Low- and High-IPI Subgroups of GOYA (E) and BC Cancer Registry (F) Cohorts



Abbreviations: ABC = activated B-cell–like; BC = British Columbia; *BCL2* = B-cell lymphoma 2; GCB = germinal center B-cell–like; IPI = International Prognostic Index; PFS = progression-free survival.

values of ≥ 50% are used,[41] which is in accordance with the recent World Health Organization criteria,[40] and suggests that applying the 50% cutoff is important for achieving high reproducibility.

Clone 124 was developed as a candidate for our clinical trial use beginning in 2011, based in large part on the 20-year history of widespread clinical use of that clone. E17 and SP66 were available

within several years of that date, but were not widely used, and their relative performance had not yet been described. Importantly, our findings support the conclusion of Schuetz et al[42]—that there is little reason to prefer E17 over 124. However, they contradict the findings of Kendrick et al,[32] who concluded that SP66 is markedly superior to either of the other two clones. Furthermore, additional studies are required to determine whether the small number of cases identified by E17 but not by clone 124 in the MAIN cohort is a reproducible finding.

Using the methodology described in this report, we demonstrated an overall incidence of *BCL2* positivity of approximately 50% across the studied cohorts, which is comparable with the prevalence estimate of around 50% reported in studies of untreated DLBCL patients.[19,20,30,43,44] The anti-*BCL2* clone 124 detected > 95% of the *BCL2*-positive cases identified by any of the *BCL2* antibodies tested. Any differences could potentially be attributed to the effect of mutations in the specific binding domain of each antibody. However, no such epitope mutations were detected in the small subset of patients with discordance in *BCL2* IHC status in this study, which is consistent with a previous observation in de novo DLBCL.[42] Furthermore, we observed broad *BCL2* expression across COO subtypes and in low- and high-IPI subgroups.

In a multivariate analysis, adjusted for IPI and COO, *BCL2* expression was associated with inferior PFS in the GOYA and BC Cancer registry patient cohorts, with a similar trend observed in the MAIN cohort. While only a subset of patients enrolled in the MAIN and GOYA studies was available for biomarker analysis, demographic data indicate that these groups were broadly representative of the overall study populations and that findings for the biomarker population may be applicable to the broader ITT population. Together, these results across clinical trials and a registry cohort confirm the relevance of the *BCL2* IHC assay to identify poor prognostic patients in newly diagnosed DLBCL.

In addition to the overall association between *BCL2* positivity and inferior PFS, we identified subsets of *BCL2*-positive patients expressing additional clinical (*BCL2* IHC-positive and IPI-high) or molecular markers (*BCL2* positive within ABC subtype, *BCL2*/*MYC* DE), which may put them at even higher risk and increased need of effective treatments. The lack of statistical significance in the ABC cohort seems more likely due to the smaller sample size (n = 249 vs. 421 in GCB), rather than to an actual difference in the effect. While it is fair to hypothesize that there might be a difference in importance of *BCL2* positivity between the GCB and ABC cohorts, a much larger sample size would be necessary to conclusively determine a difference; literature is inconsistent[20,45] and therefore cannot be used to predict which cohort is at higher risk.

Although further investigation is required, patients within these high-risk subsets may benefit from incorporation of a *BCL2*-targeted therapy such as venetoclax into their treatment regimen. In preclinical studies we have observed that *BCL2* expression is the strongest predictor of venetoclax sensitivity, whilst B-cell lymphoma−extra large (*BCL-XL*) and/or myeloid cell leukemia 1 (*MCL-1*) coexpression may confer resistance in *BCL2* high-expressing models.[28,46] These data suggest that adding *BCL-XL* and *MCL-1* expression may provide incremental specificity for venetoclax response, but would require a more complex diagnostic test. However, only clinical trial data will clarify what, if any, fraction of these patients will actually receive an advantage. Indeed, improved PFS and overall survival with venetoclax combined with R-CHOP in *BCL2*-positive patients (identified using the Ventana assay) have been reported in a phase 2 study of first-line DLBCL patients (NCT02055820).[47] Future prospective trials to determine whether *BCL2* inhibitors should be incorporated into clinical practice, and well-defined methods to characterize the target population, are needed to maximize the therapeutic potential of these novel targeted agents.

Multiple publications have evaluated the prognostic significance of *BCL2* in newly diagnosed DLBCL patients (summarized in Supplementary Table 1 in the online version). In contrast to the algorithm developed in this study, previous studies evaluating the prognostic value of *BCL2* expression in DLBCL have used variable cutoffs (from > 1% to > 75%) and did not include information on staining intensity, which may explain in part the differences in prognostic impact.[22,23] In addition to methodologic differences in the assay, patient population (age, staging and treatment, and clinical trial eligibility) and follow-up time varied between these studies. Despite these differences, multiple studies, including clinical trial and population-based registry cohorts, have demonstrated an inferior prognosis associated with *BCL2* expression in newly diagnosed DLBCL patients treated with R-CHOP, and have also confirmed prognostic impact in multivariate analyses adjusting for clinicopathologic risk factors in DLBCL. The current study includes the largest data set for R-CHOP to date and broadens the evidence that *BCL2* is an independent prognostic factor, while addressing prior concerns about the reproducibility of assessing *BCL2* routinely in the clinical setting. Furthermore, a more comprehensive understanding of the molecular pathogenesis of *BCL2* expression using a standardized assay in large clinical cohorts will lead to the investigation of more specific therapeutic strategies to mitigate the worse outcome among *BCL2*-positive patients.

In summary, we have developed a highly reproducible *BCL2* IHC scoring system and assay that incorporates a biologically defined cutoff based on sensitivity to venetoclax, and have used this assay to assess samples from 3 patient cohorts. Our findings add to the current body of evidence and confirm that *BCL2* is a negative prognostic marker in DLBCL. Furthermore, our results demonstrate that *BCL2* expression remains a poor prognostic indicator in R-CHOP-treated patients, including low- and high-IPI groups, and within COO subtypes.

Clinical Practice Points

- Conflicting results have been published on the prognostic significance of B-cell lymphoma 2 (*BCL2*) expression in patients with diffuse large B-cell lymphoma (DLBCL) after therapy with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP).
- Our analysis reports the development of a simple, reproducible scoring system and assay for *BCL2* immunohistochemistry (IHC) that included a biologically relevant cutoff for *BCL2* staining, based on venetoclax drug sensitivity. Further, using this system, we investigated the prognostic importance of *BCL2* in previously untreated patients with DLBCL who had received R-CHOP in the phase 3 MAIN and GOYA studies, and in a real-world patient registry data set from the British Columbia Cancer Agency.

- The robustness of the method was established through its high reproducibility when assessed with 3 different *BCL2* antibody clones (E17, clone 124, and SP66, using the same scoring algorithm and controlling for similar staining intensities) and interlaboratory reproducibility studies.
- *BCL2* expression was associated with poor progression-free survival in all 3 patient cohorts using a multivariate analysis that was adjusted for International Prognostic Impact (IPI) and cell-of-origin subtypes. Additionally, we identified subsets of *BCL2*-positive patients expressing additional clinical (*BCL2* IHC positive and IPI-high) or molecular markers (*BCL2* positive within activated B-cell–like subtype, *BCL2*/*MYC* double expressing), which may put them at even higher risk and increased need of effective treatments.
- Results support the use of the *BCL2* IHC scoring system and assay in future studies to identify patients with DLBCL that is *BCL2* positive, which may be particularly useful in the investigational evaluation of targeted *BCL2* inhibitors.

Acknowledgments

The phase 3 MAIN and GOYA studies, on which this analysis is based, were supported by research funding from F. Hoffmann-La Roche Ltd, Basel, Switzerland. Access to samples from BC Cancer was supported by research funding from F. Hoffmann-La Roche Ltd/Genentech Inc. The authors thank the patients and study investigators who participated in the MAIN and GOYA studies. The authors also thank Chris Bolen (Genentech Inc) for his contribution to data analysis, Mehrdad Mobasher, MD, MPH, for his contribution to the design of the analysis, development of methodology, acquisition of data, analysis and interpretation of data, and study supervision, and Jeffrey Venstrom, MD, for editorial guidance. Under the direction of the authors, editorial assistance was provided by Tara N. Miller and Tara Beers Gibson of Envision Scientific Solutions, and medical writing support was provided by Susan Hasmall and Kate Rijnen of Gardiner-Caldwell Communications, and funded by F. Hoffmann-La Roche Ltd.

Disclosure

E.P., F.V.P., E.S.-G., R.B., E.K., and A.D.D. are employees of Genentech Inc, and hold F. Hoffmann-La Roche Ltd stock. G.L. is an employee of Roche Products Ltd and holds F. Hoffmann-La Roche Ltd stock. L.Z. is an employee of Ventana Medical Systems Inc. P.F., R.D.G., and A.M. have no conflicts of interest to declare. F.J.M. and K.E.M. are employees of F. Hoffmann-La Roche Ltd and hold F. Hoffmann-La Roche Ltd stock. M.M. consults for or advises F. Hoffmann-La Roche Ltd and Janssen Pharmaceuticals, and participates in speakers' bureaus for F. Hoffmann-La Roche Ltd, Janssen Pharmaceuticals, Celgene, Gilead Sciences, Novartis, and Takeda. G.A.S. has received research support from F. Hoffmann-La Roche Ltd and honoraria from F. Hoffmann-La Roche Ltd for participation in scientific boards or intervention in symposia. L.H.S. consults for AbbVie, Amgen, Celgene, Gilead, Janssen, Lundbeck, F. Hoffmann-La Roche Ltd/Genentech Inc, Seattle Genetics, and TG Therapeutics, and has received honoraria from AbbVie, Seattle Genetics, and TG Therapeutics. J.F.S. is a member of advisory boards, and has received travel support and research support from AbbVie and F. Hoffmann-La Roche Ltd/Genentech Inc. M.T. has received honoraria, and consults or advises for AbbVie, Amgen, AstraZeneca, Bristol-Myers Squibb, Gilead Sciences, Incyte, Janssen Pharmaceuticals, MorphoSys, Portola, Takeda, and F. Hoffmann-La Roche Ltd, consults or advises for Celgene, has received travel support from AbbVie, AstraZeneca, Bristol-Myers Squibb, Gilead Sciences, Janssen Pharmaceuticals, Portola, F. Hoffmann-La Roche Ltd, and Takeda, and has received research funding from F. Hoffmann-La Roche Ltd. M.Z.O. was an employee of F. Hoffmann-La Roche Ltd during the study and document preparation, and holds F. Hoffmann-La Roche Ltd stock. U.V. consults for or advises F. Hoffmann-La Roche Ltd, Janssen Pharmaceuticals, Celgene, and Juno Therapeutics, participates in speakers' bureaus for F. Hoffmann-La Roche Ltd, Janssen Pharmaceuticals, Celgene, Gilead Sciences, and Takeda, and has received research support from F. Hoffmann-La Roche Ltd and travel support from F. Hoffmann-La Roche Ltd, Alexion Pharmaceuticals, and Janssen Pharmaceuticals.

Supplementary Data

Supplemental material, tables and figures accompanying this article can be found in the online version at https://doi.org/10.1016/j.clml.2020.11.004.

References

1. Martelli M, Ferreri AJ, Agostinelli C, et al. Diffuse large B-cell lymphoma. *Crit Rev Oncol Hematol* 2013; 87:146-71.
2. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. *Blood* 1997; 89:3909-18.
3. Anderson JR, Armitage JO, Weisenburger DD. Epidemiology of the non-Hodgkin's lymphomas: distributions of the major subtypes differ by geographic locations. Non-Hodgkin's Lymphoma Classification Project. *Ann Oncol* 1998; 9:717-20.
4. Armitage JO. My treatment approach to patients with diffuse large B-cell lymphoma. *Mayo Clin Proc* 2012; 87:161-71.
5. Gisselbrecht C, Glass B, Mounier N, et al. Salvage regimens with autologous transplantation for relapsed large B-cell lymphoma in the rituximab era. *J Clin Oncol* 2010; 28:4184-90.
6. International Non-Hodgkin's Lymphoma Prognostic Factors Project. A predictive model for aggressive non-Hodgkin's lymphoma. *N Engl J Med* 1993; 329:987-94.
7. Sehn LH, Berry B, Chhanabhai M, et al. The revised International Prognostic Index (R-IPI) is a better predictor of outcome than the standard IPI for patients with diffuse large B-cell lymphoma treated with R-CHOP. *Blood* 2007; 109:1857-61.
8. Sweetenham JW. Diffuse large B-cell lymphoma: risk stratification and management of relapsed disease. *Hematol Am Soc Hematol Educ Program* 2005:252-9.
9. Shipp MA, Ross KN, Tamayo P, et al. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. *Nat Med* 2002; 8:68-74.
10. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000; 403:503-11.
11. Lenz G, Wright G, Dave SS, et al. Stromal gene signatures in large–B-cell lymphomas. *N Engl J Med* 2008; 359:2313-23.
12. Roschewski M, Staudt LM, Wilson WH. Diffuse large B-cell lymphoma—treatment approaches in the molecular era. *Nat Rev Clin Oncol* 2014; 11:12-23.
13. Nowakowski GS, LaPlant B, Macon WR, et al. Lenalidomide combined with R-CHOP overcomes negative prognostic impact of non-germinal center B-cell phenotype in newly diagnosed diffuse large B-cell lymphoma: a phase II study. *J Clin Oncol* 2015; 33:251-7.
14. Younes A, Sehn LH, Johnson P, et al. Randomized phase III trial of ibrutinib and rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone in non-germinal center B-cell diffuse large B-cell lymphoma. *J Clin Oncol* 2019; 37:1285-95.
15. Davies A, Cummin TE, Barrans S, et al. Gene-expression profiling of bortezomib added to standard chemoimmunotherapy for diffuse large B-cell lymphoma (REMoDL-B): an open-label, randomised, phase 3 trial. *Lancet Oncol* 2019; 20:649-62.
16. Gascoyne RD, Adomat SA, Krajewski S, et al. Prognostic significance of Bcl-2 protein expression and *Bcl-2* gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. *Blood* 1997; 90:244-51.

17. Horn H, Ziepert M, Becher C, et al. *MYC* status in concert with *BCL2* and *BCL6* expression predicts outcome in diffuse large B-cell lymphoma. *Blood* 2013; 121:2253-63.
18. 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* 2003; 101:4279-84.
19. 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* 2011; 117:7070-8.
20. 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* 2011; 17:7785-95.
21. 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* 2010; 51:199-212.
22. de Jong D, Rosenwald A, Chhanabhai M, et al. Immunohistochemical prognostic markers in diffuse large B-cell lymphoma: validation of tissue microarray as a prerequisite for broad clinical applications—a study from the Lunenburg Lymphoma Biomarker Consortium. *J Clin Oncol* 2007; 25:805-12.
23. Tsuyama N, Sakata S, Baba S, et al. *BCL2* expression in DLBCL: reappraisal of immunohistochemistry with new criteria for therapeutic biomarker evaluation. *Blood* 2017; 130:489-500.
24. Davids MS, Roberts AW, Seymour JF, et al. Phase I first-in-human study of venetoclax in patients with relapsed or refractory non-Hodgkin lymphoma. *J Clin Oncol* 2017; 35:826-33.
25. Seymour JF, Pfreundschuh M, Trněný M, et al. R-CHOP with or without bevacizumab in patients with previously untreated diffuse large B-cell lymphoma: final MAIN study outcomes. *Haematologica* 2014; 99:1343-9.
26. Vitolo U, Trněný M, Belada D, et al. Obinutuzumab or rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone in previously untreated diffuse large B-cell lymphoma. *J Clin Oncol* 2017; 35:3529-37.
27. Scott DW, Mottok A, Ennishi D, et al. Prognostic significance of diffuse large B-cell lymphoma cell of origin determined by digital gene expression in formalin-fixed paraffin-embedded tissue biopsies. *J Clin Oncol* 2015; 33:2848-56.
28. Punnoose EA, Leverson JD, Peale F, et al. Expression profile of *BCL-2, BCL-XL*, and *MCL-1* predicts pharmacological response to the *BCL-2* selective antagonist venetoclax in multiple myeloma models. *Mol Cancer Ther* 2016; 15:1132-44.
29. Souers AJ, Leverson JD, Boghaert ER, et al. ABT-199, a potent and selective *BCL-2* inhibitor, achieves antitumor activity while sparing platelets. *Nat Med* 2013; 19:202-8.
30. Johnson NA, Slack GW, Savage KJ, et al. Concurrent expression of *MYC* and *BCL2* in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. *J Clin Oncol* 2012; 30:3452-9.
31. Pezzella F, Tse AG, Cordell JL, et al. Expression of the bcl-2 oncogene protein is not specific for the 14;18 chromosomal translocation. *Am J Pathol* 1990; 137:225-32.
32. Kendrick SL, Redd L, Muranyi A, et al. *BCL2* antibodies targeted at different epitopes detect varying levels of protein expression and correlate with frequent gene amplification in diffuse large B-cell lymphoma. *Hum Pathol* 2014; 45: 2144-53.
33. Adam P, Baumann R, Schmidt J, et al. The *BCL2* E17 and SP66 antibodies discriminate 2 immunophenotypically and genetically distinct subgroups of conventionally *BCL2*-"negative" grade 1/2 follicular lymphomas. *Hum Pathol* 2013; 44:1817-26.
34. Gurel B, Iwata T, Koh CM, et al. Nuclear *MYC* protein overexpression is an early alteration in human prostate carcinogenesis. *Mod Pathol* 2008; 21:1156-67.
35. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2. *Genome Biol* 2014; 15:550.
36. Sehn LH, Oestergaard MZ, Trněný M, et al. Prognostic impact of *BCL2* and *MYC* expression and translocation in untreated DLBCL: results from the phase III GOYA study. *Hematol Oncol* 2017; 35:131-3.
37. Pfeifer M, Zheng B, Erdmann T, et al. Anti-CD22 and anti-CD79B antibody drug conjugates are active in different molecular diffuse large B-cell lymphoma subtypes. *Leukemia* 2015; 29:1578-86.
38. 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* 2003; 100:9991-6.
39. Scott DW, Wright GW, Williams PM, et al. Determining cell-of-origin subtypes of diffuse large B-cell lymphoma using gene expression in formalin-fixed paraffin-embedded tissue. *Blood* 2014; 123:1214-7.
40. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood* 2016; 127:2375-90.
41. Ambrosio MR, Lazzi S, Bello GL, et al. MYC protein expression scoring and its impact on the prognosis of aggressive B-cell lymphoma patients. *Haematologica* 2019; 104:e25-8.
42. Schuetz JM, Johnson NA, Morin RD, et al. *BCL2* mutations in diffuse large B-cell lymphoma. *Leukemia* 2012; 26:1383-90.
43. Hu S, Xu-Monette ZY, Tzankov A, et al. MYC/BCL2 protein coexpression contributes to the inferior survival of activated B-cell subtype of diffuse large B-cell lymphoma and demonstrates high-risk gene expression signatures: a report from the International DLBCL Rituximab-CHOP Consortium Program. *Blood* 2013; 121:4021-31.
44. Green TM, Young KH, Visco C, et al. Immunohistochemical double-hit score is a strong predictor of outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. *J Clin Oncol* 2012; 30:3460-7.
45. 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* 2006; 24:961-8.
46. Mérino D, Khaw SL, Glaser SP, et al. Bcl-2, Bcl-x(L), and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. *Blood* 2012; 119:5807-16.
47. Morschhauser F, Feugier P, Flinn IW, et al. Venetoclax plus rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP) improves outcomes in *BCL2*-positive first-line diffuse large B-cell lymphoma (DLBCL): first safety, efficacy and biomarker analyses from the phase II CAVALLI study. *Blood* 2018; 132:782.

Supplementary Materials and Methods

Development and Validation of Fluidigm Cell-of-Origin Classifier Assay

Gene expression analysis was performed using the Fluidigm BioMark quantitative real-time PCR (qRT-PCR) platform (Fluidigm, South San Francisco, CA). Ninety-six TaqMan primer/probe sets were included (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA). All TaqMan assays in the gene expression panel were either made-to-order or custom-designed FAM minor groove binders and were ordered through Life Technologies (Carlsbad, CA, USA) (Supplementary Table 5). The geometric mean of the threshold cycle (Ct) values for 2 of the reference genes (*TMEM55B*, *VPS-33B*) was calculated for each sample, and expression levels were determined using the delta Ct (ΔCt) method as follows: Ct(Target gene) − GeoMean Ct(Reference genes). Finally, genewise expression scores for a given sample were normalized to those observed in a Human Universal Reference Total RNA sample (UHR; Clontech Laboratories, Mountain View, CA) (delta ΔCt [ΔΔCt]): ΔCt(sample) − ΔCt(UHR). We validated the Fluidigm platform demonstrating concordance between Fluidigm and Affymetrix (Santa Clara, CA). The Affymetrix platform was used to assess gene expression, given that the Fluidigm assay was developed before the availability of the cell-of-origin (COO) Nanostring assay. Correlation with COO classification by Nanostring was subsequently performed and is reported below.

For technical validation of the assay, we compared Fluidigm qRT-PCR to Affymetrix data in a cohort of 15 nontrial subjects with matched fresh-frozen (FF) and formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples.

Diffuse Large B-Cell Lymphoma Subtyping Using Fluidigm COO Classifier Assay

We adapted the linear-predictor score (LPS) classifier[E1] to the Fluidigm COO Classifier assay by recalibrating the gene-specific weights and classification boundaries. This tailored the classifier built on microarray profiling of FF diffuse large B-cell lymphoma (DLBCL) tissue to use qRT-PCR measurements from FFPE material: subsets of activated B-cell–like (ABC) and germinal center B-cell–like (GCB) reference samples in the MAIN data set were identified through unsupervised analysis of classifier gene expression (partitioning around medoids).[E2] For use as gene-specific weights, *t* statistics were obtained by comparing GCB versus ABC reference samples: *IGHM* (−7.02), *ETV6* (−5.79), *IL16* (−5.2), *IRF4* (−4.6), *SH3BP5* (−4.5), *PTPN1* (−2.42), *FUT8* (−2.19), *BLNK* (−2.07), *ENTPD1* (−1.96), *PIM1* (−1.56), *BMF* (−0.27), *DDB1* (3.65), *BCL6* (7.83), *LMO2* (8.27), *MME* (9.35), *LRMP* (9.87), *DENND3* (10.19), *NEK6* (11.82), and *MYBL1* (12.75). For each sample, the LPS was obtained as the weighted sum of the ΔΔCt expression scores across all predictor genes. LPS thresholds corresponding to a classification probability of 90% were calculated on the basis f the mean and standard deviation of the LPS of the ABC and GCB DLBCL reference samples. ABC/GCB subtypes were assigned to all samples exceeding 90% classification probability; samples with a lower classification probability (corresponding to an intermediate LPS) remained unclassified.

Supplementary Results

DLBCL Subtype

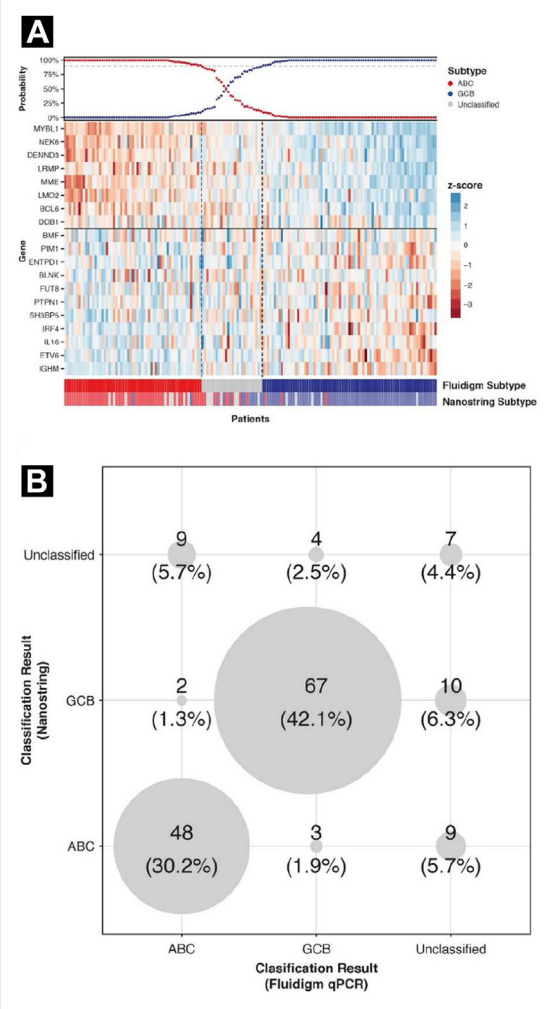
Validation of the COO assay by comparing Fluidigm qRT-PCR with Affymetrix data in a cohort of 15 nontrial subjects revealed a high correlation between qRT-PCR measurements from matched FF and FFPE samples across 19 classifier genes assessed. We also found a high correlation between Affymetrix microarray and Fluidigm qRT-PCR measurements from the same FF samples.

Weights for classifier genes calculated from qRT-PCR data from the Fluidigm COO Classifier assay were highly concordant with those obtained from previously published microarray data in an independent patient cohort.[E3]

We observed high (76%) concordance between LPS derived from the Fluidigm assay data in FFPE tumor and from Affymetrix microarray data in matched FF tissue in the technical registry cohort, applying the previously described gene expression signature.[E1]

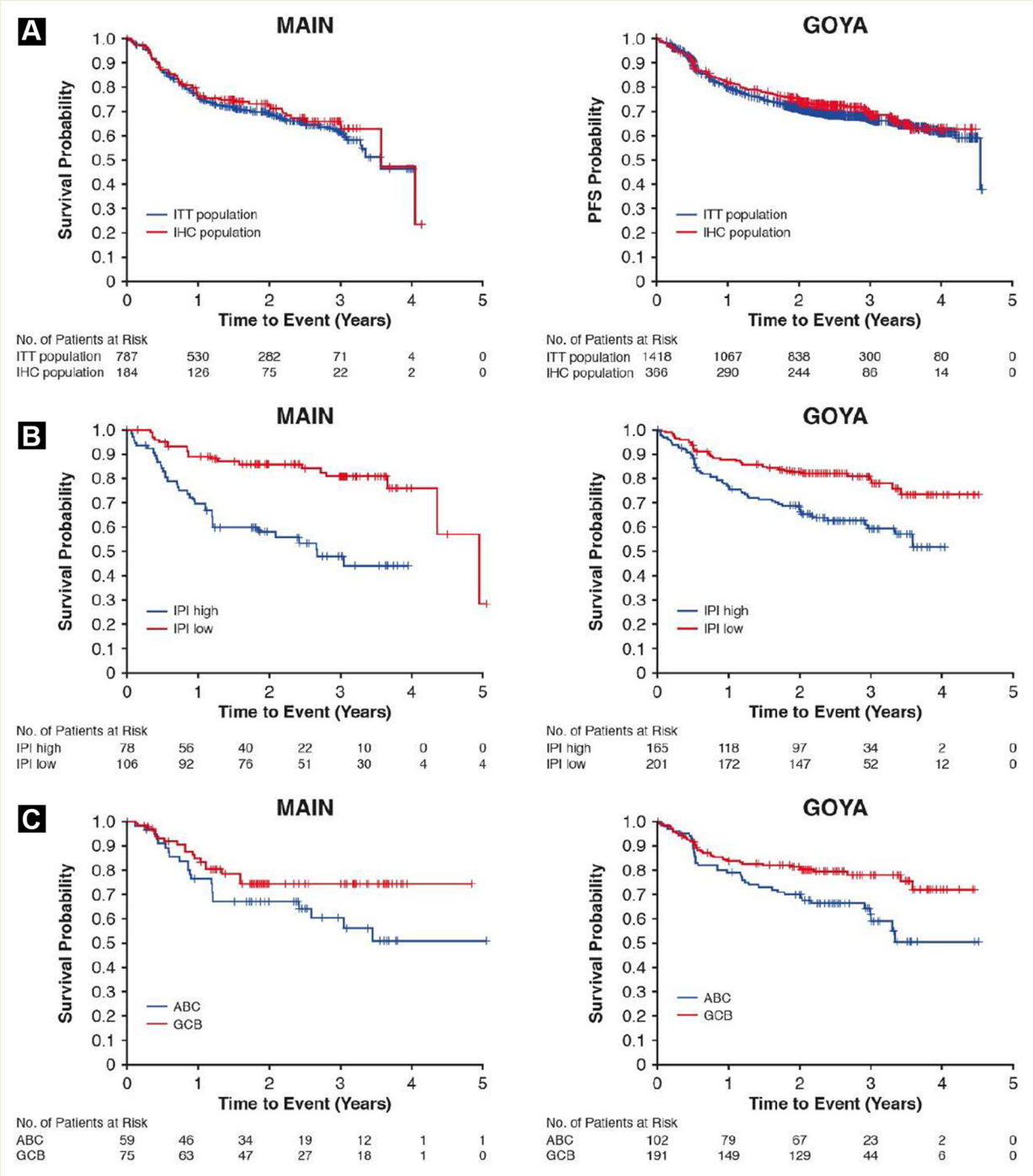
Of 160 samples from MAIN, 59 (36.9%) were ABC and 75 (46.9%) were GCB COO subtypes, while 26 samples (16.3%) remained unclassified (Supplementary Figure 1A). Lymph2Cx confirmed 48 (81.4%) of 59 of the ABC and 67 (89.3%) of 75 of the GCB classifications assigned using the Fluidigm COO Classifier assay (Supplementary Figure 1B). Most differences between the subtyping platforms occurred at the decision boundaries; for example, samples were considered unclassifiable by one method but not the other (Supplementary Figure 1B).

Supplemental Figure 1 **Distribution of COO Subtypes by Fluidigm COO Classifier Assay. (A) Unsupervised Analysis of Classifier GENE Expression Across MAIN Data Set (n = 160): > 90% Probability Threshold Was Applied. (A, B) Concordance of COO Subtype Assignment Based on (A) Fluidigm and (B) Nanostring Gene-expression Data**



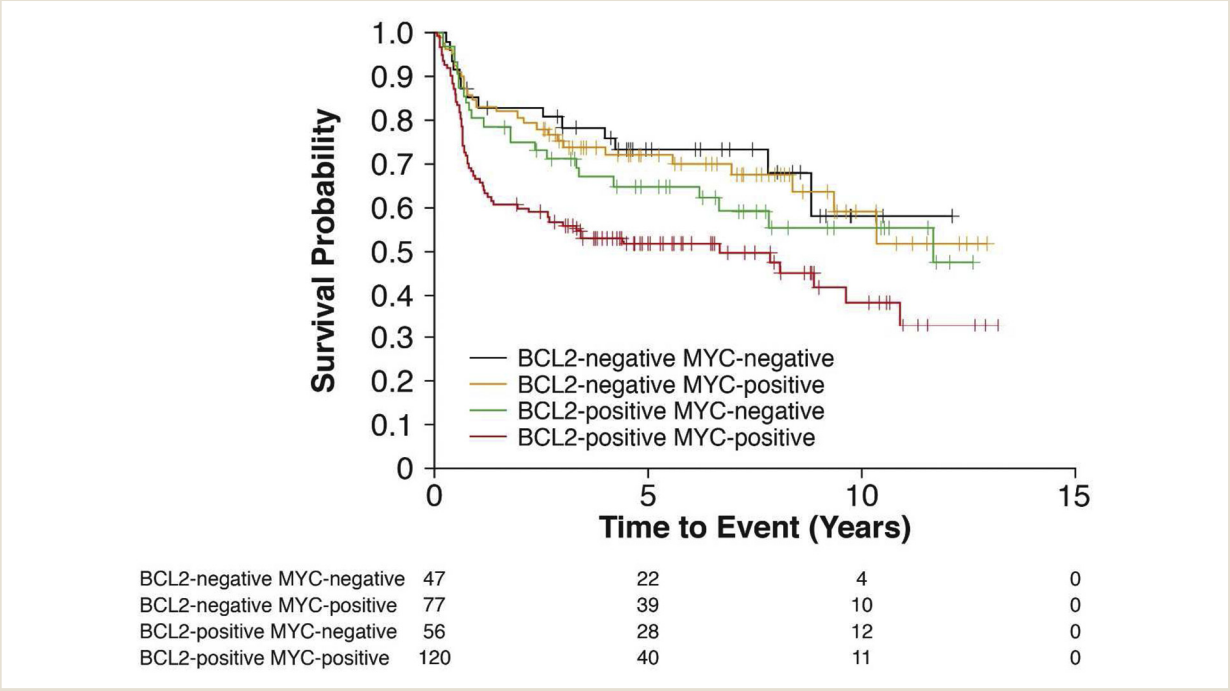
Abbreviations: ABC = activated B-cell–like; COO = cell-of-origin; GCB = germinal center B-cell–like; qPCR = quantitative polymerase chain reaction.

Supplemental Figure 2 PFS Estimates in MAIN and GOYA Studies. (A) Biomarker and Overall Populations. (B) Low- and High-IPI Subgroups (IPI-low: 0, 1, 2; IPI-high: 3, 4, 5) of the Biomarker Population. (C) ABC and GCB COO Subgroups of the Biomarker Population



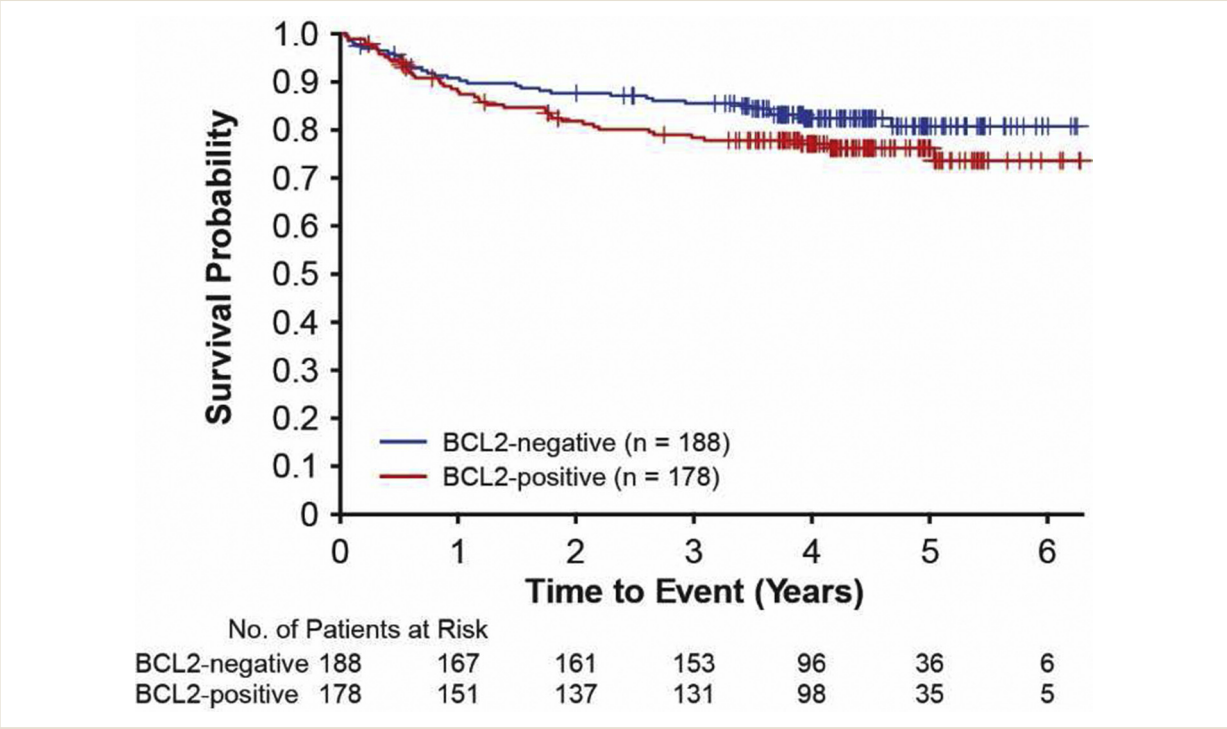
Abbreviations: ABC = activated B-cell–like; CI = confidence interval; COO = cell of origin; GCB = germinal center B-cell–like; HR = hazard ratio; IHC = immunohistochemistry; IPI = International Prognostic Index; ITT = intention to treat; PFS = progression-free survival.

Supplemental Figure 3 Kaplan-Meier Curves for PFS in *BCL2/MYC* Single-Positive, Double-Positive, and Double-Negative Patients in BC Cancer Registry Cohort



Abbreviations: BC Cancer = British Columbia Cancer; *BCL2* = B-cell lymphoma 2; PFS = progression-free survival.

Supplemental Figure 4 Kaplan-Meier Curve for OS for *BCL2*-Positive Versus *BCL2*-Negative Expression in *BCL2* IHC Populations in GOYA Cohort



Abbreviations: *BCL2* = B-cell lymphoma 2; IHC = immunohistochemistry; OS = overall survival.

Supplementary Table 1 Publications Assessing Prognostic Value of *BCL2* in First-Line DLBCL

Study	Patient Population and Treatment	No. of Patients Available for Testing	*BCL2* IHC Antibody Clone	*BCL2* IHC-Positive Definition	*BCL2*-Positive (%)	Conclusion
Gascoyne RD, *Blood* 1997[E4]	BC Cancer registry; chemotherapy regimen (MACOP-B or VACP-B)	139	Rabbit polyclonal	>10% positive	24	*BCL2* expression correlated with decreased 8-year OS (34% vs. 60%; $P < .01$), DFS (32% vs. 66%; $P < .001$), and RFS (25% vs. 59%; $P < .001$), and remained significant in multivariate analysis, including IPI ($P < .02$).
Mounier N, *Blood* 2003[E5]	GELA LNH-98—5 trial in elderly DLBCL; CHOP vs. R-CHOP	292	Clone 124	>50% positive	66	R-CHOP overcomes *BCL2*-associated resistance in elderly DLBCL patients. *BCL2* protein was prognostic in CHOP-treated patients with a decreased 2-year OS (48 ± 11% for *BCL2*-positive patients and 67 ± 14% for *BCL2*-negative patients; $P < .05$), but not for R-CHOP—treated patients (67 ± 9% for *BCL2*-positive patients and 72 ± 12% for *BCL2*-negative patients).
Salles G, *Blood* 2011[E6]	Lunenburg consortium included 12 studies from clinical trials or population-based reference centers; CHOP or CHOP-like chemotherapy ± rituximab	1305	Clone 124	>75% positive	49	Optimal cutoff for *BCL2* predicting OS of patients could only be determined in CHOP-treated patients (HR, 1.6; 95% CI, 1.1—2.3; $P = .2$) and with marginal difference i n R-CHOP-treated patients (HR, 1.4; 95% CI, 0.9—2.2; $P = .09$). A prognostic model for patients treated in the R era, which included *BCL2*, identified 4 risk groups with improved discrimination of low-risk patients, but the study concluded that IPI remained the best prognostic index for patients treated with R-CHOP.
Iqbal J, *Clin Cancer Res* 2011[E7]	LLMPP consortium; R-CHOP-treated (or CHOP-like chemotherapy) patients	169	Clone 124	≥50% positive	44	*BCL2* protein expression has significant impact on OS and EFS in DLBCL (OS, $P = .009$; EFS, $P = .001$). Within COO subtypes, *BCL2* expression impacts GCB-DLBCL (OS, $P = .03$; EFS, $P = .002$), but not ABC-DLBCL.
Johnson NA, *J Clin Oncol* 2012[E8]	R-CHOP-treated patients from BC Cancer and other institutions	304	Clone 124	≥50% positive	53	*BCL2* protein expression is prognostic in univariate analysis. *BCL2* and *MYC* coexpression "DE" remains prognostic in the multivariate model after adjusting for high-risk features.
Green TM, *J Clin Oncol* 2012[E9]	R-CHOP-treated patients identified from Danish National Lymphoma database and a multicenter cohort	193 (t), 116 (v)	Clone 124	>70% positive	50	Coexpression of *BCL2* and *MYC* protein was associated significantly with OS and PFS in the multivariate model that controlled for IPI and COO subtypes (OS, $P < .001$; PFS, $P < .001$).
Hu S, *Blood* 2013[E10]	R-CHOP-treated patients identified through International DLBCL R-CHOP Consortium Program study	466 (t), 234 (v)	Clone 124	>70% positive	50	*BCL2*/*MYC* coexpression is a strong independent predictor of OS ($P < .0001$) and PFS ($P < .0001$) in DLBCL patients in multivariate analysis controlling for other clinicopathologic features. Prognostic impact of *BCL2* expression on OS and PFS was dependent on inclusion of *BCL2*/*MYC*-coexpressing patients.
Horn H, *Blood* 2013[E11]	RICOVER-60 clinical trial of the DSHNHL; patients >60 years treated with R-CHOP	442	NA	>0%	80	*BCL2* protein expression is associated with inferior EFS (relative risk, 1.9; 95% CI, 1.0—3.8; $P = .064$) and OS (risk ratio, 2.4; 95% CI, 1.0—5.5; $P = .039$) in Cox models adjusted for IPI.

Supplementary Table 1 Continued

Study	Patient Population and Treatment	No. of Patients Available for Testing	*BCL2* IHC Antibody Clone	*BCL2* IHC-Positive Definition	*BCL2*-Positive (%)	Conclusion
Valera A, *Haematologica* 2013[E12]	Patients treated with R-CHOP or R-chemotherapy at institutions of GELCAB group	219	Clone 124	≥50% positive	58	Patients with *BCL2*-positive tumors had shorter PFS and OS than those who were *BCL2*-negative (5-year PFS 49% vs. 69%, respectively, $P = .009$; and 5-year OS 57% vs. 73%, $P = .09$). In the multivariate analysis, *BCL2*-positive patients had inferior PFS (HR, 2.1; $P = .009$), but not OS.
Scott DW, *J Clin Oncol* 2015[E13]	BC Cancer registry, patients treated with R-CHOP	344	Clone 124 and E17	≥50% positive	65	COO and *BCL2/MYC* DE are independently prognostic of each other by multivariate analysis in a 6.5-year follow-up for OS analysis.
Petrella T, *Ann Oncol* 2017[E14]	LYSA clinical trial LNH03–6B; in elderly patients treated with R-CHOP	285	Clone 129	≥70% positive	61	*BCL2* expression predicted worse PFS (HR, 1.79; $P = .003$) and OS (HR, 1.67; $P = .02$) independently of IPI score.
Tsuyama N, *Blood* 2017[E15]	Japanese Foundation for Cancer Research population-based registry; patients treated with R-CHOP	218 (t), 238 (v)	Clone 124	≥90% positive at staining intensity ≥T cells	40	A new *BCL2* scoring system was developed based on % positivity and intensity. *BCL2*-positive status (IHC 3+) was a significant prognostic factor independent of IPI, IHC based COO and *MYC* protein/rearrangement status in a training set (n = 218) for PFS (HR, 3.84; 95% CI, 2.21–6.67; $P < .001$) and OS (HR, 2.13; 95% CI, 1.21–3.75; $P < .009$). Adverse prognostic effect was confirmed in a validation set (n = 238).
Staiger AM, *J Clin Oncol* 2017[E16]	Updated analysis of RICOVER-60 clinical trial of the DSHNHL; patients >60 years treated with R-CHOP; and R-Mega CHOEP trial in patients ≤ 60 years age-adjusted IPI 2,3	RICOVER-60 R-CHOP cohort: 168; R-Mega CHOEP cohort: 88	NA	>50%	62[a]	No difference in EFS, PFS, or OS between ABC vs. GCB by multivariate analysis. Patients with DE status had significantly inferior survival compared with non-DEs ($P < .001$ for each outcome of EFS, PFS, and OS) in the R-CHOP–treated GCB DLBCL group but not in the ABC group. *BCL2* expression indicated a trend toward an inferior outcome within GCB DLBCL, but not within ABC DLBCL.

Abbreviations: ABC = activated B-cell–like; BC Cancer = British Columbia Cancer; CHOEP = cyclophosphamide, doxorubicin, etoposide, vincristine, prednisone; CHOP = cyclophosphamide, doxorubicin, vincristine, prednisone; CI = confidence interval; COO = cell of origin; DE = double expressor; DFS = disease-free survival; DLBCL = diffuse large B-cell lymphoma; DSHNHL = Die Deutsche Studiengruppe für Hochmaligne Non-Hodgkin-Lymphome; EFS = event-free survival; GCB = germinal center type B-cell–like; GELCAB = Grup per l'Estudi dels Limfomes de Catalunya i Balears; HR = hazard ratio; IHC = immunohistochemistry; IPI = International Prognostic Index; LLMPP = Leukemia and Lymphoma Molecular Profiling Project; LYSA = Lymphoma Study Association; MACOP-B = methotrexate with leucovorin rescue, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin; NA = not applicable; OS = overall survival; PFS = progression-free survival; R = rituximab; RFS = relapse-free survival; t = training cohort; v = validation cohort; VACP-B = etoposide, doxorubicin, cyclophosphamide, vincristine, prednisolone, bleomycin.

[a]Data are provided for the RICOVER-60 R-CHOP cohort.

Supplementary Table 2 Non-Hodgkin Lymphoma Cell Lines

Cell Line	Venetoclax IC_{50} (μM)	*BCL2* IHC Score
Ri-1	0.01	3+
OCI-LY19	0.02	3+
Sc-1	0.03	3+
RL	0.09	3+
DoHH2	0.13	2+
SU-DHL 4	0.16	3+
SU-DHL-6	0.16	2+
DB	0.22	3+
WSU-FSCCL	0.35	3+
Granta-519	0.61	3+
Rec-1	1.19	1+
WSU-DLCL2	2.48	3+
Karpas 422	3.00	3+
SU-DHL-5	3.00	3+
Jeko-1	3.00	1+
MC116	3.00	1+
Ci-1	3.00	1+
Karpas-1106p	3.00	1+
MHH-PREB1	3.00	1+
BJAB	3.00	0
Ramos	3.00	0
Scc-3	3.00	0
Su-DHL 10	3.00	0
SU-DHL-1	3.00	0
SU-DHL8	3.00	0
U698M	3.00	0

Abbreviations: IC_{50} = half-maximal inhibitory concentration; IHC = immunohistochemistry.

Supplementary Table 3 Antibody Clone Information for MAIN, GOYA, and BC Cancer Registry Studies

Study	Antibody Used	Antibody Version
MAIN	Anti-*BCL2* (clone 124) mouse model antibody	Research only
	Anti-*BCL2* clone E17 (epitope 61—76 amino acids)	Research only
	Anti-*BCL2* SP66 rabbit monoclonal antibodies	Research only
	MYC clone Y69 Epitomics antibody	Research only
GOYA[a]	Anti-*BCL2* (clone 124) mouse model antibody	Investigational
	MYC clone Y69 Epitomics antibody	Investigational
BC Cancer registry[a]	Anti-*BCL2* (clone 124) mouse model antibody	Investigational
	MYC clone Y69 Epitomics antibody	Investigational

Abbreviations: BC Cancer = British Columbia Cancer; *BCL2* = B-cell lymphoma 2.
[a]Studies assessed later, after the investigational use assay became available.

Supplementary Table 4 Patient Baseline Demographics and Characteristics for Biomarker Populations Versus All Patients Enrolled Onto MAIN and GOYA Studies

Characteristic	MAIN		GOYA	
	Biomarker (N = 230)	All Patients (N = 787)	*BCL2*-evaluable (N = 366)	All Patients (N = 1418)
Sex				
Female	53	51	52	47
Male	47	49	48	52
Age (years)				
Median (range)	62 (20–79)	61 (18–89)	63 (20–83)	62 (18–86)
Race				
Asian	5	20	15	37
White	77	69	80	60
Other	18	11	5	3
ECOG PS				
0–1	73	80	86	87
2–3	26	20	14	13
Missing	1	<0.5	<0.5	<0.5
Bulky (≥7.5 cm) disease				
Yes	43	50	36	37
No	57	50	64	63
IPI score				
0–2	59	57	55	55
3–5	41	42	45	45
Unknown	0	1	0	0
Ann Arbor stage				
I-II	29	26	27	24
III-IV	70	72	73	76
Unknown	1	2	0	<0.5
Treatment group				
Control	49	50	50	50
Experimental	51	50	50	50

Data are presented percentages unless otherwise indicated.
Abbreviations: *BCL2* = B-cell lymphoma 2; ECOG PS = Eastern Cooperative Oncology Group performance status; IPI = International Prognostic Index.

Supplementary Table 5 Oligonucleotides Used to Detect DLBCL Classifier Genes, *BCL2*, and 2 Housekeeping Genes

Gene Name	Gene Type	Life Source Catalog No.
BCL6	Classifier	Hs00277037_m1
BLNK	Classifier	Hs00179459_m1
BMF	Classifier	Hs00372937_m1
DDB1	Classifier	Hs01096550_m1
DENND3	Classifier	Hs00392099_m1
ENTPD1	Classifier	Hs00969559_m1
ETV6	Classifier	Hs00231101_m1
FUT8	Classifier	Hs00189535_m1
IGHM	Classifier	Hs00941538_g1
IL16	Classifier	Hs00189606_m1
IRF4	Classifier	Hs01056533_m1
LMO2	Classifier	Hs00153473_m1
LRMP	Classifier	Hs00153480_m1
MME	Classifier	Hs00153510_m1
MYBL1	Classifier	Hs00277143_m1
NEK6	Classifier	Hs00205221_m1
PIM1	Classifier	Hs00171473_m1
PTPN1	Classifier	Hs00182260_m1
SH3BP5	Classifier	Hs00183137_m1
TMEM55B	Housekeeping	Hs00292741_m1
VPS33B	Housekeeping	Custom design[a]
BCL2		Custom design[b]

Abbreviations: DLBCL = diffuse large B-cell lymphoma; FAM = 6-fluorescein amidite.
[a]FAM probe sequence, and forward and reverse primer sequences: TGGAGCAGCTTCCT, GGCTCGAGACCAGCTCATCTA, and GAGATCTGCCTCAATGAATAAATCC, respectively.
[b]FAM probe sequence, and forward and reverse primer sequences: TGCACACCTGGATCC, CCTGTGGATGACTGAGTACCTGAA, and GGGCCGTACAGTTCCACAAA, respectively.

Supplementary References

E1. 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* 2003; 100:9991-6.

E2. Reynolds AP, Richards G, de la Iglesia B, et al. Clustering rules: a comparison of partitioning and hierarchical clustering algorithms. *J Math Model Algorithms* 2006; 5:475-504.

E3. Lenz G, Wright G, Dave SS, et al. Stromal gene signatures in large–B-cell lymphomas. *N Engl J Med* 2008; 359:2313-23.

E4. Gascoyne RD, Adomat SA, Krajewski S, et al. Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. *Blood* 1997; 90:244-51.

E5. 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* 2003; 101:4279-84.

E6. 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* 2011; 117:7070-8.

E7. 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* 2011; 17:7785-95.

E8. Johnson NA, Slack GW, Savage KJ, et al. Concurrent expression of *MYC* and *BCL2* in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. *J Clin Oncol* 2012; 30:3452-9.

E9. Green TM, Young KH, Visco C, et al. Immunohistochemical double-hit score is a strong predictor of outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. *J Clin Oncol* 2012; 30:3460-7.

E10. Hu S, Xu-Monette ZY, Tzankov A, et al. *MYC*/*BCL2* protein coexpression contributes to the inferior survival of activated B-cell subtype of diffuse large B-cell lymphoma and demonstrates high-risk gene expression signatures: a report from the International DLBCL Rituximab-CHOP Consortium Program. *Blood* 2013; 121:4021-31.

E11. Horn H, Ziepert M, Becher C, et al. *MYC* status in concert with *BCL2* and *BCL6* expression predicts outcome in diffuse large B-cell lymphoma. *Blood* 2013; 121:2253-63.

E12. Valera A, Lopez-Guillermo A, Cardesa-Salzmann T, et al. *MYC* protein expression and genetic alterations have prognostic impact in patients with diffuse large B-cell lymphoma treated with immunochemotherapy. *Haematologica* 2013; 98:1554-62.

E13. Scott DW, Mottok A, Ennishi D, et al. Prognostic significance of diffuse large B-cell lymphoma cell of origin determined by digital gene expression in formalin-fixed paraffin-embedded tissue biopsies. *J Clin Oncol* 2015; 33:2848-56.

E14. Petrella T, Copie-Bergman C, Briere J, et al. *BCL2* expression but not *MYC* and *BCL2* coexpression predicts survival in elderly patients with diffuse large B-cell lymphoma independently of cell of origin in the phase 3 LNH03–6B trial. *Ann Oncol* 2017; 28:1042-9.

E15. Tsuyama N, Sakata S, Baba S, et al. *BCL2* expression in DLBCL: reappraisal of immunohistochemistry with new criteria for therapeutic biomarker evaluation. *Blood* 2017; 130:489-500.

E16. Staiger AM, Ziepert M, Horn H, et al. Clinical impact of the cell-of-origin classification and the *MYC*/ *BCL2* dual expresser status in diffuse large B-cell lymphoma treated within prospective clinical trials of the German high-grade non-Hodgkin's lymphoma study group. *J Clin Oncol* 2017; 35:2515-26.