BCL2 Predicts Survival in Germinal Center B-cell-like Diffuse Large B-Cell Lymphoma Treated with CHOP-like therapy and Rituximab

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Translation relevance

Patients with diffuse large B-cell lymphoma (DLBCL) can be divided into two major molecular subgroups: activated-B-cell like (ABC)-DLBCL and germinal-center B-cell like (GCB)-DLBCL. BCL2 is a major anti-apoptosis factor and we have found that BCL2 expression is associated with poorer outcome in the ABC but not GCB cases in patients treated with CHOP (cyclophosphamide, vincristine, doxorubicin, and prednisone). The introduction of rituximab (R) in CHOP-like therapies in DLBCL may alter the prognostic significance of previously studied biomarkers. We re-evaluated BCL2 as a prognosticator and found that BCL2 expression is now associated with poorer outcome in GCB-DLBCL as BCL2-positive GCB-DLBCL has shown less improvement with R-CHOP compared with the BCL2 negative counterpart. Our study demonstrated the important change in BCL2 as a prognosticator in the setting of R-CHOP and suggests that BCL2 positive GCB-DLBCL cases may benefit from novel agents such as inhibitors of BCL2 function.
**Purpose:** We have previously demonstrated the prognostic significance of BCL2 expression in the activated B-cell-like diffuse large B-cells lymphoma (ABC-DLBCL) patients treated with CHOP or CHOP-like therapy. However, after the inclusion of rituximab in the CHOP regimen, several conflicting observations regarding the prognostic value of BCL2 expression have been reported.

**Patient/Methods:** We evaluated the R-CHOP cohort of 221 DLBCL cases with gene expression profiling data. BCL2 protein (n=169), mRNA (n=221) expression and t(14;18) (n=144) were correlated with clinical outcome. The CHOP-cohort (n=181) was used for comparative analysis.

**Results:** BCL2 protein expression has significant impact on overall-survival (OS) and event-free-survival (EFS) in DLBCL (OS p=0.009, EFS p=0.001) and GCB-DLBCL (OS p=0.03, EFS p=0.002), but not in ABC-DLBCL in the R-CHOP cohort. These survival differences were also observed in multivariate analysis. At the mRNA level, this correlation was observed in EFS in DLBCL (p=0.006), but only a trend was observed in GCB-DLBCL (p=0.09). The t(14;18) was detected in 35% of GCB-DLBCL but was not associated with significant differences in survival. Gene-enrichment analysis identified significant enrichment of the DLBCL “stroma-1” signatures and HIF1-α signature in BCL2(-)GCB-DLBCL, while TFH cell signatures was enriched in BCL2(+)GCB-DLBCL.

**Conclusion:** The prognostic significance of BCL2 has changed after inclusion of rituximab in the treatment protocol, and is observed in the GCB-DLBCL rather than the ABC-DLBCL. Although rituximab has benefitted patients in both DLBCL subgroups, the BCL2 (+)GCB-DLBCL appears to receive less benefit from this treatment and may require other novel therapeutic intervention.
Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma (NHL), with several morphologic and clinicopathologic variants. Distinctive molecular and genetic abnormalities have been identified in DLBCL, and patients with this disease exhibit a wide range of clinical presentations and outcomes. The International Prognostic Index (IPI) is a widely-accepted tool used to predict the clinical outcome of the patients with DLBCL. However, new therapeutic regimens with better effectiveness can alter the significance of prognostic markers and modifications of the IPI have recently been proposed to better predict the outcome in DLBCL.

Gene expression profiling (GEP) studies in CHOP-era have shown that patients with DLBCL derived from germinal center B-cells (GCB-DLBCL) have a better survival than patients those with DLBCL derived from activated B-cells (ABC-DLBCL). However, the addition of the anti-CD20 antibody rituximab has revolutionized the treatment of DLBCL, leading to a significant increase in survival, but survival advantage in patients with the GCB-DLBCL persists.

BCL2 functions as an anti-apoptotic factor and is frequently deregulated in DLBCL. One well-characterized mechanism of BCL2 overexpression is the t(14;18)(q32;q31), which is largely restricted to GCB-DLBCL whereas, ABC-DLBCL the mechanism of BCL2 overexpression is associated with constitutive NF-κB activation, with or without 18q21 amplification. BCL2 has been extensively studied as a prognostic biomarker in DLBCL, albeit with controversial findings which were thought to be due to the heterogeneity within DLBCL. Our previous studies of DLBCL patients treated with CHOP-like therapies have demonstrated that BCL2 expression has prognostic significance in the ABC-DLBCL, but not in GCB-DLBCL.

Since the standard treatment of DLBCL now includes rituximab, several studies with conflicting observations concerning prognostic value of BCL2 expression in R-CHOP-treated patients have been reported in recent years with some showing and some not showing any prognostic
significance. Therefore, we analyzed our cohort of GEP-defined DLBCL to determine if BCL2 overexpression still has prognostic value in patients with DLBCL treated in the R-CHOP era.

**Materials and Methods**

**Patient information**

Two-hundred and twenty-one cases of *de novo* DLBCL treated with rituximab and CHOP or CHOP-like therapies were obtained from the LLMPP consortium (3). The CHOP- cohort (n=181) was used for comparative analysis (3, 12) only. This study was approved by the institutional review boards of the respective institutions, and all patients gave written informed consent.

**BCL2 Immunohistochemical evaluation**

Of the R-CHOP cohort, 169 cases were evaluated for BCL2 protein expression, using BCL2 antibody (Clone-124, Dako-Carpinteria, CA) as reported previously (12, 26). Approximately half of the cases (TMAs) had been scored previously by two pathologists (WC and DDW) (27) and these cases were re-scored by (PNM) with no significant disagreement. The rest of the cases were then evaluated by PNM and his scores were used for this study. The tumor cell percentage was recorded in 10% increments, and ≥50% staining was considered positive. The optimal cut-point for BCL2 expression (positive vs negative in survival-analysis) was determined by survival-tree methods using p-values from adjusted log-rank statistics. The adjusted log-rank statistics takes into account that a large number of cut-points may be tested when determining the optimal cut-point(28). In this approach, optimal cut point for biomarker is selected as the one with the maximum adjusted log-rank statistics to predict patient OS in all data, and then this cut point is applied for subgroups analysis. The survival trees were created using the “party” package: A Laboratory for Recursive Partytioning in R (29, 30).

**BCL2 mRNA expression**
GeneChip HG-U133 plus2.0 array (Affymetrix Inc., Santa Clara, CA) was previously used for GEP for determining the cell-of-origin in both R-CHOP and CHOP cohort (3). *BCL2* mRNA expression was measured from mean-intensity of 3 probe-sets (232210_at, 244035_at, 232614_at) out of the 8 probe-sets present on HG-U133 plus2.0 array. These probe-set were chosen based on their consistence in measurement, the signal intensity and their ability to measure *BCL2* transcript from cases with *BCL2* rearrangement.

**Detection of the t(14;18)(q32;q21) by fluorescence in-situ hybridization (FISH) and copy number changes**

Of the R-CHOP cohort, 144 cases were evaluated for presence of t(14;18)(q32;q21) by interphase FISH using dual color “break-apart” probes (Abbott Molecular, Abbott Park, IL) as previously described (10). The break-apart signals in >5% cells were considered “positive” for translocation. The presence of three or > four signals of 18q21 along with two signals for Centromere-18 was considered as a gain and amplification respectively.

**Statistical analysis**

Chi-square test, Spearman correlation-coefficient and Wilcoxon rank was used to determine the association between categorical variables, *BCL2* mRNA/protein and between *BCL2* mRNA/protein and *BCL2* rearrangement data respectively. The Kaplan-Meier method was used for overall survival (OS) and event-free survival (EFS) analysis as done previously (12). Cox-regression was used in multivariate modeling of OS and EFS data for both the BCL2 protein and mRNA data, after adjusting for IPI and GEP classification. SAS software V 9.2 (SAS Institute Inc., Cary, NC) was used for all analyses other than creation of the survival trees.

BRB-ArrayTools (version-3.7.0) (31) and GSEA (32) computational programs were used to identify differentially-expressed genes, and pathways/signatures between BCL2–positive and -negative group in GCB-DLBCL.
Results

Patient characteristics

We examined BCL2 mRNA expression levels in 221 DLBCL cases in the R-CHOP cohort including GCB-DLBCL (n =102, 46%), ABC-DLBCL (n =88, 40%), and unclassifiable DLBCL (n =31, 14%), with protein expression and BCL2 translocation data available in 169 and 144 cases respectively. A flow-chart outlining the number of patients in each DLBCL subgroups for mRNA, protein, t(14;18) analysis is shown in Figure1. The clinical features at the time of presentation for the R-CHOP cohort (n=221) were not significantly different, when compared to the CHOP cohort (n=180) we had studied previously (12) (Supplemental Table-1), except for better OS (p<0.001) in the R-CHOP cohort. As expected, the cell-of-origin (p<0.005) and the IPI (p<0.0001) were both independent predictors of OS in each cohort (Supplemental Figure-1).

There were significant differences in some of the clinical features associated with BCL2 protein expression, in DLBCL as a single entity with a higher median-age and stage seen in the BCL2-positive group (Table-1), but no differences were observed with regard to mRNA expression (Supplemental Table-2). Among the cell-of-origin subgroups, there were no differences in clinical features in the ABC-DLBCL cases with regard to BCL2 protein expression. However, in the GCB-DLBCL cases, a higher median-age, stage, LDH levels and IPI scores were in the BCL2 protein positive group (Table-1), whereas no differences were seen with regard to mRNA expression (Supplemental Table-2).

Occurrence of the t(14;18) and correlation of BCL2 mRNA and protein expression

The t(14;18) was observed in 19% (27 of 144) of the DLBCL cases and 34% (22 of 64) of GCB-DLBCL cases, consistent with our previous finding in the CHOP cohort(10). However, four cases with the t(14;18) occurred in the ABC-DLBCL. Interestingly, further analysis of these four cases showed c-MYC rearrangement (double hit) in two and BCL2 amplification (>4 copies) in the other two cases.
BCL2 protein expression was observed in 44% (75 of 169) of the DLBCL, and was more frequent in ABC-DLBCL (62%, 45 of 73) compared to GCB-DLBCL (30%, 22 of 73) (p=0.0002). A similar trend was observed at the mRNA level as well, with significantly higher expression observed in ABC-DLBCL (Table-2). We also observed a significant association between BCL2 mRNA and protein expression in DLBCL as a group, and in both the ABC and GCB subgroups (Spearman correlation 0.64, p<0.0001). However, there were a number of discrepant cases (3 cases (of 42) in lowest mRNA quartile showed high (≥50%) protein expression, and 8 (of 43) cases in highest mRNA quartile showed low (<50%) protein expression, that may be due to methodological limitations or biological variance such as differences in post-transcriptional regulation of BCL2 expression or variations due to stromal components expressing BCL2. The t(14;18) was also significantly associated with an increased BCL2 protein expression (p<0.0001) and mRNA level (p<0.0001) in GCB-DLBCL (Supplemental Table-3). However two GCB-DLBCL cases with the t(14;18) were negative for protein expression and showed very low mRNA expression, whereas six cases without the t(14;18) showed high BCL2 mRNA and protein expression.

The evaluation of BCL2 copy number changes revealed a significant association of 18q21 amplification (>3 copies, p=0.01) with ABC-DLBCL (12 amplified and 8 gain; of 31 cases) compared with GCB-DLBCL (3 amplified and 4 gain; of 29 cases). Interestingly, all three cases of GCB-DLBCL with an amplified 18q21 locus were negative for BCL2 protein expression and had low mRNA expression whereas, in ABC-DLBCL, the majority of cases (10 of 12) with an amplified 18q21 locus were positive for protein expression and showed high mRNA expression (Supplemental Figure-2).

Prognostic significance of BCL2 expression

BCL2 protein expression by immunostaining with 50% cutoff for positive cases, was significantly associated with OS (p = 0.009) or EFS (p =0.001) in the entire cohort of DLBCL patients (Figure-2). To further substantiate this finding, we added 62 DLBCL cases that were entered into the study but did not have acceptable GEP data, to the 169 cases with GEP data for a total of 231 cases. Similar results were obtained.
with both OS (p=0.005) and EFS (p<0.001), thus providing confirmation that BCL2 is a prognostic marker in R-CHO- treated patients (Supplemental Figure-2).

Because mRNA expression is a continuous variable, we divided the patients arbitrarily into two or four groups according to the BCL2 transcript levels. When DLBCL was analyzed as a single entity, patients with high BCL2 mRNA levels had a significantly worse EFS regardless of whether cases were divided into two groups (p=0.006), or quartiles (p=0.036), but no significant difference was observed in OS (Figure-3).

When the prognostic significance of BCL2 expression was analyzed for the DLBCL cell-of-origin, we observed strikingly different results compared to our previously-studied CHOP-cohort (12). There is now no significant association of either BCL2 protein or mRNA expression and survival in ABC-DLBCL (Figure-4), whereas a significant association of OS (p=0.03) and EFS (p=0.002) with BCL2 protein expression is now observed in GCB-DLBCL (Figure-5). At the mRNA level, no significant association was observed with OS or EFS in GCB-DLBCL, but a trend was observed in EFS. This trend was more prominent when GCB-DLBCL cases were divided into quartiles (Supplemental Figure-3).

The multivariate analysis of OS and EFS in DLBCL as a single entity showed that BCL2 protein was a marginal significant predictor of OS (HR=2.0, 95% CI: 1.0-4.0; p=0.06) and a significant predictor of EFS (HR=2.0, 95% CI: 1.1-3.6; p=0.02) independent of IP1. However BCL2 mRNA expression was a marginal significant predictor of EFS (p=0.06), but not OS (p=0.15). Similar analysis in GCB-DLBCL subgroup showed that protein expression was predictive of EFS (HR=4.5, 95% CI: 1.2-16.5; p=0.02), but not OS(HR=3.0, 95% CI: 0.6-15.8) after adjusting for IPI. However, effective sample size for this analysis was small (n=59).

**Differential gene expression between BCL2 protein positive and negative groups in GCB-DLBCL**

More than 500 transcripts were differentially expressed (>1.5 fold and p<0.005) between the BCL2-positive and negative cases in the GCB subgroup (Figure-6). As expected BCL2 transcripts were highly expressed in the BCL2-positive cases, but some pro-apoptotic genes e.g. BCL2L1(BIM) were also highly
expressed. Approximately half of the transcripts upregulated in the BCL2-positive cases were uncharacterized. However, the informative ones included a heterogeneous group that has been recently associated with B-cell neoplasms including FOXP2(33), CEACAM1(34), CLLU1(35) and AKT2(36). Many of these genes either promote survival or regulate B-cell signaling. Interestingly, a large number of genes overexpressed in the BCL2-negative group were involved in cell adhesion or regulating the extracellular matrix (DSG2, GJB2, CLDN1, NRXN3, PARD3, and CADM1). When the significance level of the t-test was lowered to p=0.01 for differential expression, the majority of genes involved in cell cycle progression and regulation were upregulated in the BCL2-negative groups, similar to our previous findings(10). In contrast, genes involved in apoptosis (BAD, BAK1, BTG1, and BNIP3L) and BCR signaling (BCAP29, BLK, LYN) or mainly B-cell related (FCAR, FCRL1, FCRL2) were more prominent in the BCL2-positive group. GSEA analysis identified enrichment of the proliferation signature, dendritic cell (resting signature), DLBCL stromal-1 signature, HIF1α regulated gene signature and normal mesenchymal signature in the BCL2-negative group (Figure-6). These observations are consistent with previous findings that the stromal-1 signature is significantly associated with HIF1-α (37), normal mesenchymal signature, and histiocytes and can predict better OS(3). The BCL2-positive group did not show any significantly enriched pathways with the exception of the T_{FH} signature, suggesting increased infiltration of T_{FH}-cells in these cases.
Discussion

BCL2 regulates programmed cell-death and plays an important role in the response of malignant cells to a variety of stresses that lead to apoptosis, including chemotherapy (38). The association of BCL2 expression with survival in DLBCL patients treated with CHOP or CHOP-like regimens had conflicting results, with studies showing either significant or no significant association with OS (13-19). We have previously shown that the prognostic significance of BCL2 expression in the context of DLBCL cell-of-origin, which may explain many of these conflicting findings (12). We and others have shown that ABC-DLBCL patients treated with CHOP-like therapies have a shorter OS if the BCL2 is overexpressed (12, 20) which may be due to the ability of these tumor cells to resist apoptosis induced by chemotherapy, and showed mechanistic differences in BCL2 upregulation in the GCB and ABC subgroups (10).

The addition of rituximab to CHOP-like protocols for DLBCL has led to a significant (p<0.001) increase in patient survival and such regimens are now considered the standard of care for DLBCL (39, 40). Several recent studies of R-CHOP cohorts have failed to show a BCL2 prognostic effect due to a disproportionate benefit from rituximab of BCL2-positive cases, and with no association of BCL2 expression with OS in DLBCL (23-25). Similarly, a patient cohort treated with rituximab and EPOCH also showed no association of BCL2 with OS or EFS (41). However, other studies have shown a prognostic influence of BCL2 mRNA (42) or protein expression in R-CHOP-treated cohorts (43-45). Song et al (22) reported significant correlation of BCL2 protein expression with OS in GCB-like DLBCL, whereas Nyman et al showed significance in non-GCB-DLBCL, only marginal (p=0.07) in GCB like-DLBCL (43). Another report of R-CHOP cohort has indicated that BCL2 protein expression influences relative risk (RR) in OS (RR: 2.3 P=0.06) and EFS (RR=2.2, p=0.03) in BCL6-positive DLBCL, but not in BCL6 negative cases (46) which is significantly associated with non-GCB-DLBCL. The conflicting reports about the prognostic significance of BCL2 expression in the literature can partly be attributed to (i) heterogeneity of the DLCBL cases studied with different proportion of GCB and ABC DLBCL cases, (iii) patient population with different risk factors other than DLBCL subtype distinction, and (iii) variables in management (iv) technical
factors affecting immunostaining, (v) experience and subjectivity of the pathologist scoring the cases. We had discussed some of these issues in our previous study (12), and suggested that biomarkers should be evaluated in context of molecular subgroups. The heterogeneity within DLBCL has usually been addressed by immunohistochemically-defined subgroups, but this approach had not been very consistent in defining prognostic groups among various laboratories due mainly to (iv) and (v) discussed above. Different algorithms have also been created by evaluating the expression of several antigens and used as surrogate for GEP-based classification. Interestingly, when we evaluated these algorithms against GEP defined subtypes, most of these performed very well with all the immunostains performed in one laboratory (47), unlike a recent study, where none of the algorithms showed any prognostic significance(48). Since GEP-defined molecular subgroups remain the gold standard for DLBCL classification, we have used GEP-defined subgroups in our study to avoid all the variables from immunohistochemical classification from influencing our results.

In this study, tumors were considered positive, if at least 50% of neoplastic cells show BCL2 expression. The 50% cut-off value has been frequently but not consistently used in assessing BCL2 positivity. The optimal cut point was, therefore, chosen using survival tree method (28) as an unbiased approach using p-values from adjusted log rank statistics. A similar approach has been been used recently in large DLBCL series for different biomarkers cut-points(49). Our study shows that when DLBCL patients are treated with rituximab and CHOP-like therapies, cases that overexpresses BCL2 protein have significant poorer OS (p=0.009) and EFS (p=0.001). This association was observed at the mRNA level for EFS as well. Similar to our previous findings in CHOP cohort(12), there were also significant differences in other basic clinical features observed in the BCL2 protein positive and negative groups in this R-CHOP series as well, suggesting that BCL2 expression may be associated with age and stage, but these differences were not observed at the mRNA level. Likewise IPI score, Karnofsky score and stage showed significant differences between BCL2 protein positive and negative group in GCB-DLBCL, but not at mRNA level. Consistent with our previous observations and other reports, BCL2-positive cases are observed more
frequent in ABC-DLBCL than in GCB-DLBCL (10, 22, 43). However, the expression of BCL2 mRNA or protein expression was not predictive of either OS or EFS in ABC-DLBCL in our R-CHOP cohort, unlike our CHOP cohort reported previously (12). In contrast, we have now observed a significant association of BCL2 protein expression with poor OS and EFS in GCB-DLBCL and this was observed at the mRNA level with marginal significance in EFS only (Supplemental Figure-3). The association of BCL2 with survival was independent of other clinical features as shown in multivariate analysis. Therefore, we conclude that BCL2-positive ABC-DLBCL has benefited proportionally more from R-CHOP than BCL2-negative tumors, narrowing the differences in survival in this group. However, this is not true for GCB-DLBCL, with BCL2-positive tumors benefiting less from this treatment compared with BCL2-negative tumors, thus resulting in a significant difference in patient survival. Although rituximab was reported to significantly improve the outcome of patients with BCL2-positive DLBCL, a distinction was not made between the subtypes (50).

Our findings raise an important consideration regarding the interaction of rituximab with the two DLBCL- cell-of-origin subgroups. In ABC-DLBCL, overexpression of BCL2 protein is associated with constitutive activated NF-κB pathway(11) and 18q21 amplification(12). In vitro experiments have shown that rituximab down regulates NF-κB and its target BCL2 (51) and it may be similarly effective in reducing the expression of BCL2 in vivo, resulting in increased susceptibility to chemotherapy. On the other hand, rituximab may fail to down modulate BCL2 in GCB-DLBCL, which contributes to drug resistance in this subset of tumors. If this is indeed correct, then additional measures that reduce BCL2 levels or function may be effective in improving survival in this group of GCB-DLBCL patients. BCL2 may also interact with other oncogenic pathways as shown by the consistently poor prognosis in patients with high expression of both BCL2 and c-MYC genes (52). Since BCL6 is almost universally expressed by GCB-DLBCL and can repress genes that control DNA damage response(53) and check point p53, ATR (54, 55), it may synergize with high BCL2 expression in resisting drug-induced apoptosis in GCB-DLBCL.

One unexpected observation in our series was that the t(14;18) was not predictive of OS or EFS in GCB-DLBCL, even-though it was highly associated with BCL2 expression. This may be due to subthreshold
(<50%) protein expression in 36% of the t(14;18) positive cases and the expression of BCL2 protein (>50%) in 8% of the GCB-DLBCL cases lacking this translocation, thus reducing the correlation between BCL2 translocation and survival.

To investigate further the mechanisms that may play a role in the difference in survival between the BCL2- positive and -negative GCB-DLBCL groups, we analyzed the differences in GEP between these two groups. We found that the stromal-1 signature was associated with the BCL2-negative group (3). The “stromal-1” signature is related to extracellular-matrix deposition and mesenchymal and histiocytic cell infiltration, and is associated with favorable outcome (3). The BCL2-negative group was also associated with higher proliferation, confirming what we observed previously (10). The BCL2-positive group did not show any significantly enriched gene expression signatures, except T<sub>FH</sub>-cell related signatures, which suggest the presence of increased infiltrating of T<sub>FH</sub>-cells. A recent observation showed that adhesion of lymphoma cells to follicular-dendritic cells (FDC) can protect the neoplastic cells against apoptosis or promote drug-resistance by inducing miR-181a expression resulting in down regulating BIM protein level(56). Similar protective signal may be provided by T<sub>FH</sub>-cells that are often associated with FDCs to tumor cell in contact. BIM downregulation and BCL2 expression may provide synergistic resistance to therapy.

In summary, our study have demonstrated the importance of reevaluating prognostic factors in the setting of new therapeutic regimens, and provided a new perspective in BCL2 expression and prognosis with respect to the cell-of-origin classification of DLBCL. Our findings indicated clear improvement in outcome on the use of rituximab with CHOP in ABC-DLBCL and the BCL2 protein negative subset of GCB-DLBCL. However, the BCL2-positive subset of GCB-DLBCL has shown less improvement and these cases may benefit from novel agents such as inhibitors of BCL2 function.
Acknowledgments

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**Author contribution**

JI and WCC: designed, performed study, supervised all aspects of the research and analysis, wrote and finalized the manuscript.

PNM, DWW and TCG: were responsible for design of the study, pathology review and scoring immunohistochemical stains and for the final approval of the manuscript.

LS was responsible for the statistical analysis section in the study.

NAJ performed FISH and assisted in manuscript writing.

JMC, LMS, LR, EJ, AR, GO, EC, RMB, JRC, RRT and RDG were involved in the design of the study, pathology review and provided the DLBCL cases for the study.

JMV, JMC and JOA reviewed the clinical aspects and writing of manuscript.
References


**Figure Legend**

Figure-1: Flow-chart outlining the number of patients in each analysis

Figure-2: Correlation of BCL2 protein expression with OS and EFS in R-CHOP: (A) BCL2 protein expression is significantly correlated with Overall Survival (OS) and event free survival (EFS) in the R-CHOP cohort.

Figure-3: Correlation of BCL2 mRNA with OS and EFS in R-CHOP. BCL2 mRNA shows significant correlation with EFS in DLBCL (A) Cases divided into two halves (B) cases divided into quartiles according to BCL2 mRNA expression.

Figure-4: Association of BCL2 protein and mRNA with OS and EFS in ABC-DLBCL: (A) No significant correlation at (A) protein level or (B) mRNA level.

Figure-5: Correlation of BCL2 protein and mRNA with OS and EFS in GCB-DLBCL: (A) Significant correlation in at (A) protein level; (B) marginally at mRNA level in EFS.

Figure-6: Differential expression of genes (A) and GSEA (B) between BCL2 protein positive and negative groups in GCB-DLBCL. GSEA analysis identified enrichment of gene signatures (p<0.01) in BCL2-positive and negative GCB-DLBCL groups. The enrichment score curves were obtained from GSEA software. Vertical black lines indicate the position of the enriched genes (Hit) comprising the gene set. The graph on the bottom of each panel shows the ranked list metric (signal- to-noise ratio) for each gene as a function of the rank in the ordered data set (see Subramanian et al. for more details)
Table 1: Clinical features according to BCL2 protein expression group in the R-CHOP treated patients

<table>
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<th>Protein status</th>
<th>DLBCL (n=169)</th>
<th>ABC (n=73)</th>
<th>P-value</th>
<th>GCB (n=73)</th>
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<td>28 (37%)</td>
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<td>47 (63%)</td>
<td>50 (53%)</td>
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<td>26 (58%)</td>
<td>15 (54%)</td>
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<td>45 (70%)</td>
<td>71 (81%)</td>
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<td>16 (62%)</td>
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<td>19 (30%)</td>
<td>17 (19%)</td>
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<td>11 (30%)</td>
<td>10 (38%)</td>
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<td>28 (38%)</td>
<td>52 (57%)</td>
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<td>18 (41%)</td>
<td>9 (33%)</td>
<td>0.52</td>
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<td>III/IV</td>
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<tr>
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<td>45 (62%)</td>
<td>40 (43%)</td>
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<td>26 (59%)</td>
<td>18 (67%)</td>
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<td>26 (46%)</td>
<td>48 (61%)</td>
<td>0.11</td>
<td>14 (41%)</td>
<td>9 (39%)</td>
<td>0.98</td>
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<td>30 (54%)</td>
<td>31 (39%)</td>
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<td>20 (59%)</td>
<td>13 (61%)</td>
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<td>19</td>
<td>15</td>
<td></td>
<td>11</td>
<td>6</td>
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<tr>
<td>Number of extranodal sites</td>
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<td>60 (88%)</td>
<td>77 (88%)</td>
<td>0.89</td>
<td>37 (88%)</td>
<td>23 (82%)</td>
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<td>8 (12%)</td>
<td>11 (12%)</td>
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<td>5 (12%)</td>
<td>5 (18%)</td>
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<td>6</td>
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<td>33 (63%)</td>
<td>54 (73%)</td>
<td>0.26</td>
<td>21 (68%)</td>
<td>10 (48%)</td>
<td>0.15</td>
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<tr>
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<tr>
<td></td>
<td>19 (37%)</td>
<td>20 (27%)</td>
<td></td>
<td>10 (32%)</td>
<td>11 (52%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>20</td>
<td></td>
<td>14</td>
<td>7</td>
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</table>
Table 2: Expression of (A) BCL2 protein and (B) BCL2 mRNA in DLBCL subgroups of R-CHOP treated patients

(A)

<table>
<thead>
<tr>
<th>DLBCL subgroups</th>
<th>BCL2 Positive (n=); (%)</th>
<th>BCL2 Negative (n=); (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC (n=73)</td>
<td>45 (62)</td>
<td>28 (38)</td>
</tr>
<tr>
<td>GCB (n=73)</td>
<td>22 (30)</td>
<td>51 (70)</td>
</tr>
<tr>
<td>Unclassifiable (n=23)</td>
<td>8 (35)</td>
<td>15 (65)</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>DLBCL subgroups</th>
<th>Mean intensity value (log2 Scale) (SD; range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC (n=88)</td>
<td>9.1 (1.3; 5.8-11.6)</td>
</tr>
<tr>
<td>GCB (n=102)</td>
<td>8.6 (1.5; 4.8-11.8)</td>
</tr>
<tr>
<td>Unclassifiable (n=31)</td>
<td>8.7 (0.9; 7.5-11.1)</td>
</tr>
</tbody>
</table>
Figure 1: Flow-chart outlining the number of patients in each analysis

**R CHOP cohort (n=221)**
- **BCL2 mRNA** by HG-U133plus-2 (Affymetrix, Inc) microarray (n=221)
  - ABC=88
  - GCB=102
  - Unclassifiable=31

**CHOP cohort (n=181)**
- ABC=74
- GCB=76
- Unclassifiable=31

- **BCL2 protein** by Immunohistochemistry (Clone 124) (n=169)
  - ABC=73
  - GCB=73
  - Unclassifiable=23

- **t(14;18) by FISH** (dual color "break-apart" probe) (n=144)
  - ABC=63
  - GCB=64
  - Unclassifiable=17

- **62 additional R-CHOP treated DLBCL cases**

- **All three data sets available (n=128)**
  - ABC=55
  - GCB=59
  - Unclassifiable=14
Figure 2: BCL2 protein expression and overall survival (OS) and event-free survival (EFS)

OS

EFS

BCL2 protein expression

p=0.009

p=0.001

n= 94

n= 74

<50%

≥50%
Figure 3: BCL2 mRNA is correlated with EFS in DLBCL

(A)

OS

EFS

BCL2 mRNA expression
Lower half  111
Upper half  110

P=0.2

Years

0 1 2 3 4 5 6 7 8 9 10 11

Years

0 1 2 3 4 5 6 7 8 9 10 11

p=0.006

(B)

OS

EFS

Quartile
Low  1st  55
2nd  55
3rd  56
High  4th  56

p=0.4

p=0.036

Years

0 1 2 3 4 5 6 7 8 9 10 11

Years

0 1 2 3 4 5 6 7 8 9 10 11
Figure 4: BCL2 protein (A) and mRNA (B) expression is not correlated with OS and EFS in ABC-DLBCL.

(A) OS

Proportion

Years

BCL2 protein expression

<50% 28

≥50% 45

p=0.4

(B) OS

Proportion

Years

BCL2 mRNA median expression

Lower half 44

Upper half 44

p=0.52

(EFS)

Proportion

Years

p=0.4

p=0.52

p=0.14
Figure 5: BCL2 protein (A), but not mRNA (B) expression is correlated with OS and EFS in GCB-DLBCL

(A) OS

BCL2 protein expression

<50 51

>50 22

p=0.03

BCL2 mRNA mean expression

Lower half 51

Upper half 51

p=0.5

p=0.1

(B) EFS

p=0.002

p=0.5

p=0.1
Figure 6: Differential expression of genes (A) and GSEA (B) in BCL2 protein positive vs negative groups in GCB-DLBCL

(A) BCL2 Negative vs BCL2 Positive

- F2RL2
- PER5
- BCL2A1
- MCL2
- ZC3H12D
- IARS
- SM7
- FAF1
- NEDD4L
- CDH1
- CEB1
- HIF1α regulated

(B) BCL2 Positive vs BCL2 Negative

- FUC2
- VPS13C
- SCMAA5
- SGR1
- CT01
- AD1
- HIF1α regulated

HIF1α regulated

DLBCL Stromal-1 signature

- MTIP3
- BCR1
- APK14
- DCP1
- HIF1α regulated

Proliferation signature

- AKT2
- AKT2
- ZNF238
- DNAJB8
- FAM53B
- FOXP2
- PDE9P1
- TCF3
- TCF3
- EIF5B
- HNRNPD
- IARS
- GJB2
- SMG7
- DSG2
- TERF2
- FAT3
- INTS4
- NEDD4L
- CLDN1
- NRXN3
- CDS1
- CADM1
- FUCA2
- EGLN3
- VPS37C
- SCARA5
- SLC44A3
- CPXM1
- GNA11
- TEAD1
- PARD3
- EML1
- HSPA2
- PDPN

T<sub>FM</sub> – cell signature

GSEA (B) in BCL2 protein positive vs negative groups in GCB-DLBCL
BCL2 Predicts Survival in Germinal Center B-cell-like Diffuse Large B-Cell Lymphoma Treated with CHOP-like therapy and Rituximab

Javeed Iqbal, Paul N. Meyer, Lynette Smith, et al.

Clin Cancer Res  Published OnlineFirst September 20, 2011.

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Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2011/09/20/1078-0432.CCR-11-0267.DC1 http://clincancerres.aacrjournals.org/content/suppl/2011/12/12/1078-0432.CCR-11-0267.DC2

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