11-15-2017

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HHS Public Access

Author manuscript

Clin Cancer Res. Author manuscript; available in PMC 2018 November 15.

Published in final edited form as:

Clin Cancer Res. 2017 November 15; 23(22): 7119–7129. doi:10.1158/1078-0432.CCR-17-1144.

Non-Hodgkin and Hodgkin lymphomas select for overexpression of BCLW

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Abstract

Purpose—B-cell lymphomas must acquire resistance to apoptosis during their development. We recently discovered BCLW, an anti-apoptotic BCL2 family member thought only to contribute to spermatogenesis, was overexpressed in diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma. To gain insight into the contribution of BCLW to B-cell lymphomas and its potential to confer resistance to BCL2 inhibitors, we investigated the expression of BCLW and the other anti-apoptotic BCL2 family members in six different B-cell lymphomas.

Experimental Design—We performed a large-scale gene expression analysis of data sets comprising approximately 2300 lymphoma patient samples, including non-Hodgkin and Hodgkin lymphomas as well as indolent and aggressive lymphomas. Data were validated experimentally with qRT-PCR and immunohistochemistry.

Results—We report BCLW is significantly overexpressed in aggressive and indolent lymphomas, including DLBCL, Burkitt, follicular, mantle cell, marginal zone, and Hodgkin lymphomas. Notably, BCLW was preferentially overexpressed over that of BCL2 and negatively correlated with BCL2 in specific lymphomas. Unexpectedly, BCLW was overexpressed as frequently as BCL2 in follicular lymphoma. Evaluation of all five anti-apoptotic BCL2 family members in six types of B-cell lymphoma revealed that *BCL2*, *BCLW*, and *BCLX* were consistently overexpressed, whereas *MCL1* and *A1* were not. Additionally, individual lymphomas frequently overexpressed more than one anti-apoptotic BCL2 family member.

Conclusions—Our comprehensive analysis indicates B-cell lymphomas commonly select for BCLW overexpression in combination with or instead of other anti-apoptotic BCL2 family members. Our results suggest BCLW is likely equally as important in lymphomagenesis as BCL2 and that targeting BCLW in lymphomas should be considered.

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Author contributions

C.M.A, R.M., and C.M.E. designed the study; C.M.A. performed the qRT-PCR experiments; R.M. performed the bioinformatic analyses; J.Z.G. provided patient samples and evaluated the IHC; C.M.A., R.M., and C.M.E. wrote the manuscript; and all authors read and approved the manuscript.

Keywords

BCLW; BCL2; BCLX; MCL1; A1; Lymphoma

Introduction

Lymphomas, as with all human cancers, select for alterations that inhibit apoptosis, allowing them to survive during transformation (1). The BCL2 family of proteins consists of anti-apoptotic (BCL2, BCLX/BCL2L1, BCLW/BCL2L2, MCL1, and A1/BFL1) and pro-apoptotic proteins that regulate cell death (2) and have been linked to many human cancers, including lymphomas.

BCL2 translocation to the immunoglobulin locus, leading to BCL2 overexpression, is a defining feature of the non-Hodgkin indolent lymphoma follicular lymphoma (FL) (3). Diffuse large B-cell lymphoma (DLBCL), an aggressive lymphoma, can also overexpress BCL2 (4). Thirty percent of *de novo* DLBCL have BCL2 translocations and/or increased protein expression (5), correlating with reduced survival (4). In contrast to FL and DLBCL, Burkitt lymphoma, an aggressive lymphoma, express low/undetectable levels of BCL2, which is part of its diagnostics (6). We recently discovered that Burkitt lymphomas frequently overexpress BCLW, an anti-apoptotic BCL2 family member that was initially reported to only function in spermatogenesis (7,8). Targeting BCLW with shRNA or pharmacologically with BH3-mimetics induced apoptosis in Burkitt lymphoma cell lines, indicating BCLW was required for its survival (9). When overexpressed, BCLW conferred resistance to BH3-mimetics (9). We also showed that DLBCL frequently overexpresses BCLW alone or in combination with BCL2, and that increased levels of *BCLW* in patient samples with low *BCL2* expression correlated with reduced patient survival (9). Therefore, BCLW is a previously unappreciated, significant contributor to Burkitt lymphoma and DLBCL, but its involvement in other B-cell lymphomas is unknown.

There are multiple different aggressive and indolent B-cell lymphoma subtypes, but the contribution of BCLW and other anti-apoptotic BCL2 family members to them is unclear. It is reported that there is increased expression of BCL2 and BCLX in a small subset of mantle cell lymphoma (an aggressive lymphoma), whereas MCL1 expression is typically low (10). BCL2 levels can be elevated in marginal zone lymphomas, which are indolent (4). Increased levels of BCLX were identified in the majority of Hodgkin lymphomas, but are not part of its diagnostics (11). Therefore, unlike FL and DLBCL, other B-cell lymphomas have not been linked to alterations in specific anti-apoptotic BCL2 family members, which may be due to the lack of a comprehensive analysis of these genes/proteins in these lymphomas. Here, we evaluated gene expression-profiling data sets of six B-cell lymphomas for the expression of BCLW and the other anti-apoptotic BCL2 family members and validated the results with qRT-PCR and immunohistochemistry of patient samples. Our data show BCLW is frequently overexpressed in all six B-cell lymphomas, suggesting BCLW has an equally important role in lymphomagenesis as BCL2. Additionally, most of the lymphomas analyzed overexpressed more than one anti-apoptotic BCL2 family member. Our results suggest that B-cell lymphomas rely on more than one anti-apoptotic BCL2 family member for their

survival. With targeted-therapies against anti-apoptotic BCL2 family members currently being tested or developed and therapeutic resistance emerging, our data provide critical information that should have significant clinical implications.

Materials and Methods

Microarray gene expression analysis

Thirty-seven microarray gene expression-profiling data sets for six different B-cell lymphomas (Burkitt, DLBCL, follicular, mantle cell, marginal zone, and Hodgkin) and normal B-cells (germinal center centroblasts and centrocytes, memory B-cells, and CD19+ B-cells) were downloaded from the Gene Expression Omnibus (GEO) database (12) or the authors' website (6) (summarized in Supplementary Table 1). The data were generated on the Affymetrix Human Genome U133 Plus 2.0 platform following standard Affymetrix protocol. The data were separated into batches, normalized using the robust multi-array average (RMA) algorithm (13) in the Affy package for R (14). To limit/eliminate confounding, we corrected batch effects using ComBat considering two covariates (lymphoma and normal) (15). Probe sets were averaged to obtain a single-expression intensity measure per gene per array if multiple probe sets corresponded to the same gene ID. Expression values from replicate samples were averaged. Differential expression was measured using unpaired, one-tailed or two-tailed *t*-tests as indicated. All analyses were carried out in R, version 3.2.3.

Survival analysis

Gene expression profiles and patient clinicopathological information for DLBCL were extracted from the GEO database (GSE10846 and GSE31312). Data were normalized and probe sets averaged as explained above. Patient samples were simultaneously stratified based on the median expression of *BCL2* and *BCLW* into high or low expression groups for each gene. Then, patients with low *BCL2* expression were identified and Kaplan-Meier overall survival curves plotted for the high and low *BCLW* expression groups and compared using log-rank test.

Patient sample acquisition

Patient samples of formalin fixed, paraffin embedded (FFPE) sections were obtained from archival pathological specimens at Thomas Jefferson University with approval from the Institutional Review Board. Thirty follicular lymphoma, 10 marginal zone lymphoma, 14 mantle cell lymphoma, 26 Hodgkin lymphoma, and 12 normal lymph nodes were identified by a board certified hematopathologist (JZG) and processed as described below for evaluation.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from 8 10-μm sections of FFPE tissue using the RecoverAll Total Nucleic Acid Isolation Kit as per manufacturer's instructions (Thermo Fisher). cDNA was generated using SuperScript III First-Strand Synthesis (Thermo Fisher) and SybrGreen (SA-Biosciences) assays were performed in triplicate to measure mRNA expression as previously described (16). mRNA expression was normalized to *β-ACTIN* and presented as $2^{-\Delta\Delta Ct}$

comparing the mean expression of specific mRNA in lymphoma to the mean of this mRNA in the controls to determine fold-change. We previously reported the primer sequences for *BCLW*, *BCL2*, *BCLX*, and *MCL1* (17). Primers for *BCL2A1* are forward-5′-CCCGGATGTGGATACCTATAAGGAGA and reverse-5′-GTCATCCAGCCAGATTTAGGTTCA.

Immunohistochemistry (IHC)

IHC for BCL2 and BCLW was performed on 4μm FFPE sections from lymphoma and control tissues following standard procedures. BCL2 (SP66) and BCLW (ab38629, Abcam, 1:125 dilution) staining was performed using automated immunohistochemical stainers (Ventana Medical Systems and DAKO Autostainer Plus, respectively) according to manufacturer specifications. Positive and negative controls were included. Protein levels were scored by a practicing, board-certified hematopathologist (JZG) on a 0–3 scale in a blinded fashion (representative images in Supplementary Fig. 1).

Statistics

When comparing two groups, two-tailed *t*-tests were used. For gene expression, qRT-PCR, and IHC graphs, lines represent the mean ± SEM and symbols represent individual samples. For box and whisker plots, boxes represent the 25^{th} and 75^{th} percentiles, lines indicate the median, circles indicate the mean, and whiskers are the maximum and minimum. Survival analyses were compared by log-rank tests. Pearson's correlation coefficient and Spearman's rank correlation coefficient determined the expression correlation between two genes. $P<0.05$ was considered statistically significant unless otherwise noted.

Results

BCLW is overexpressed in activated B-cell (ABC) and germinal center B-cell (GCB) subtypes of DLBCL

Recently, after analyzing microarray data sets containing 57 Burkitt and 319 DLBCL patient samples, we reported that *BCLW* is frequently overexpressed in both of these lymphomas (9). Since RNA-sequencing data sets were limited, we obtained additional microarray data sets of DLBCL and normal human B-cells and combined them with those we previously analyzed. Assessing 1490 DLBCL samples showed that *BCLW* and *BCL2* were significantly overexpressed compared to normal B-cells (Fig. 1A). These results independently validate our previous study and further indicate BCLW is overexpressed in aggressive lymphomas, such as DLBCL.

DLBCL has two subtypes, ABC and GCB (18). We evaluated both subtypes for *BCLW* and as a control, *BCL2* expression. As previously reported, ABC DLBCL had significantly increased *BCL2* levels compared to normal B-cells (Fig. 1B and ref. (19)). *BCL2* levels were also increased in GCB DLBCL, but not to the same extent. In contrast, *BCLW* was equally overexpressed in ABC and GCB DLBCL (Fig. 1B). Further evaluation of ABC DLBCL showed that those with higher *BCL2* levels had lower *BCLW* levels and those with lower *BCL2* levels had higher *BCLW* levels. This inverse association between *BCLW* and

BCL2 in ABC DLBCL was significant, but not in GCB DLBCL (Fig. 1C, Supplementary Table 2, and Supplementary Fig. 2A).

Using two independent data sets containing survival data, we previously reported DLBCL patients with high levels of *BCLW* and low levels of *BCL2* had poor survival compared to patients whose DLBCL was low for both *BCLW* and *BCL2* (9). To assess whether this was specific for ABC or GCB DLBCL, we evaluated overall survival of each subtype based on *BCLW* and *BCL2* expression. Although statistical significance was not reached, both data sets showed a potential for reduced overall survival for patients with high *BCLW* and low *BCL2* in GCB, but not in ABC (Fig. 1D and Supplementary Fig. 2B). Together, our data show *BCLW* is overexpressed in both GCB and ABC DLBCL, and may affect the survival of GCB DLBCL.

Indolent lymphomas also select for BCLW overexpression

To ascertain whether selection for BCLW overexpression only occurs in aggressive lymphomas, we also evaluated indolent lymphomas. Assessment of patient samples of follicular lymphoma (FL; n=196) showed *BCL2* was characteristically overexpressed (Fig. 2A). However, there was also significant overexpression of *BCLW* compared to normal B-cells (Fig. 2A). Notably, there was no significant inverse association between *BCLW* and *BCL2* expression (Supplementary Fig. 3A), suggesting that *BCL2* overexpression was not preferentially selected over *BCLW*. In support of this, of the lymphomas that highly overexpressed *BCLW* (top 25%, n=49), 53.1% also overexpressed *BCL2* (Fig. 2B). Therefore, *BCLW* overexpression is selected for even when *BCL2* is overexpressed, suggesting BCLW is important in FL.

To determine whether the bioinformatic analyses accurately reflected expression in patients, we first performed qRT-PCR on patient samples of FL compared to normal lymph nodes. *BCL2* was significantly overexpressed (Fig. 2C and Supplementary Fig. 3B). *BCLW* was also significantly overexpressed with 93.3% (28 of 30) of the FL expressing at least four-fold more *BCLW* than normal lymph nodes (Fig. 2C and Supplementary Fig. 3B). Furthermore, consistent with previous reports (20), high-grade FL showed decreased *BCL2* expression compared to low-grade (Fig. 2D and Supplementary Fig. 3C). However, a similar level of *BCLW* overexpression was selected for regardless of grade (Fig. 2D and Supplementary Fig. 3C).

We next performed immunohistochemistry (IHC) to assess whether the mRNA levels detected were reflected at the protein level. BCLW protein was significantly overexpressed compared to normal lymph node (Fig. 2E). Specifically, BCLW staining (≥1 pathologist score) was present in all 30 FL assessed, and two-thirds received a score of 2 or 3 (Fig. 2E). Characteristically, for FL (21), nearly all showed increased BCL2 levels (Fig. 2E). There was a positive correlation between our qRT-PCR and IHC results (Fig. 2F), providing additional support that *BCLW* and *BCL2* mRNA expression is reflected at the protein level. Together, these results indicate BCLW is overexpressed in FL and that it is overexpressed at a similar frequency as BCL2.

Next, we evaluated 148 patient samples of marginal zone lymphoma (MZL), an indolent lymphoma, and determined that both *BCLW* and *BCL2* were overexpressed compared to normal human B-cells (Fig. 3A). Notably, there was a significant inverse association between *BCLW* and *BCL2* expression (Fig. 3B, Supplementary Table 2, and Supplementary Fig. 4A). Measurement of *BCLW* and *BCL2* levels by qRT-PCR in patient samples of nodal MZL also showed that both were significantly overexpressed compared to normal lymph nodes (Fig. 3C and Supplementary Fig. 4B). Although the average and median expression of *BCLW* was higher than that of *BCL2*, the negative correlation between the two was not statistically significant, likely due to our limited sample set (n=10). Evaluation of BCLW protein by IHC showed it was consistently overexpressed, as we detected high levels in MZL (average score of 2.2 out of 3). In contrast, although BCL2 protein levels were overexpressed compared to normal lymph nodes, BCL2 protein (average score of 1.7 out of 3) was not as frequently overexpressed as BCLW (Fig. 3D), indicating that BCLW may be preferentially overexpressed in nodal MZL over BCL2. A positive correlation was observed between the qRT-PCR and IHC analyses for BCLW and BCL2, indicating protein and mRNA levels were consistent (Fig. 3E). Therefore, BCLW is overexpressed in indolent and aggressive lymphomas.

Mantle cell lymphomas also overexpress BCLW

We evaluated BCLW expression in the aggressive lymphoma mantle cell lymphoma (MCL) to determine how widespread BCLW overexpression was. Assessment of 196 MCL samples showed significantly increased levels of *BCLW* and *BCL2* compared to normal human B-cells (Fig. 3F). There was no inverse association between *BCLW* and *BCL2* expression (Supplementary Fig. 5A). qRT-PCR analysis of patient samples showed that *BCLW* and *BCL2* were both significantly overexpressed in MCL compared to normal lymph nodes (Fig. 3G and Supplementary Fig. 5B). IHC for BCLW and BCL2 showed increased levels (average score of 2.2 and 2.6, respectively, out of 3; Fig. 3H). Furthermore, the mRNA expression data and IHC results were positively correlated for both BCLW and BCL2 (Fig. 3I). Therefore, our data indicate BCLW overexpression is widespread in aggressive and indolent non-Hodgkin B-cell lymphomas.

BCLW is overexpressed in Hodgkin lymphomas

To determine whether BCLW overexpression was limited to only non-Hodgkin B-cell lymphomas, we evaluated 180 Hodgkin lymphoma (HL) samples. HL showed *BCLW* overexpression compared to normal B-cells (Fig. 4A and Supplementary Fig. 6A). Also, the HL with higher *BCLW* levels had lower *BCL2* levels and those with higher *BCL2* had lower *BCLW* levels. There was a statistically significant inverse association between their expression (Fig. 4B, Supplementary Table 2, and Supplementary Fig. 6A). We evaluated patient samples of both nodular sclerosis and mixed cellularity HL by qRT-PCR. Although qRT-PCR verified the bioinformatic analyses, showing statistically significant increased expression of *BCLW* and *BCL2* mRNA, *BCLW* was overexpressed significantly more than *BCL2* in both subtypes (Fig. 4C; Supplementary Fig. 6B and 6C). The preferential selection for BCLW overexpression was also observed at the protein level, as 14 of 15 HL samples expressed high levels (pathologist score of 2 or 3) of BCLW protein, whereas only 1 sample received a high score for BCL2 protein (Fig. 4D). The results of our protein and mRNA

analyses were consistent, producing statistically significant positive correlations for both BCLW and BCL2 (Fig. 4E). Therefore, HL frequently overexpresses BCLW, and preferentially selects for BCLW overexpression over that of BCL2.

Combined, the data show that BCLW is not only overexpressed in Burkitt (9) and DLBCL, but it is also overexpressed in follicular, mantle cell, marginal zone, and Hodgkin lymphomas. Therefore, BCLW appears to contribute to both indolent and aggressive and non-Hodgkin and Hodgkin B-cell lymphomas. Importantly, the data show some lymphomas preferentially select for overexpression of BCLW instead of BCL2.

Lymphomas select for the overexpression of multiple anti-apoptotic BCL2 family members

Since we detected increased *BCLW* expression across all B-cell lymphoma subtypes analyzed (Fig. 5A) and there appeared to be preferential BCLW expression in specific subtypes, we questioned the expression of the other anti-apoptotic BCL2 family members and whether one or more would preferentially be expressed in particular lymphoma subtypes. To address this, we evaluated *BCLX*, *MCL1*, and *A1* expression in the six major human B-cell lymphomas (Burkitt, DLBCL, FL, MCL, MZL, and HL). *BCLX* was significantly overexpressed in all six lymphomas compared to normal human B-cells with the mean expression highest in DLBCL and lowest in follicular (Fig. 5B). The mean expression of *MCL1* and *A1* was significantly downregulated in Burkitt lymphoma, but showed no difference in DLBCL, FL, MZL, MCL, and HL compared to normal B-cells (Fig. 5C and 5D).

To confirm our bioinformatic results, we performed qRT-PCR on our FL patient samples for *BCLX*, *MCL1*, and *A1*. *BCLX* was significantly overexpressed compared to normal B-cells, but *MCL1* and *A1* were not (Fig. 5E and Supplementary Fig. 7A-C). However, some individual FL overexpressed *MCL1* or *A1* (Supplementary Fig. 7B and 7C). Overall, *BCLW*, *BCL2* and *BCLX*, but not *MCL1* or *A1*, were consistently overexpressed in the lymphomas analyzed.

We next assessed whether negative correlations existed between any two anti-apoptotic BCL2 family members in the six types of lymphomas. *BCLX*, *MCL1*, and *A1* showed no significant negative correlations with other anti-apoptotic BCL2 family members with three exceptions. *BCL2* was significantly negatively correlated with *MCL1* and *BCLX* in MZL, and *BCLW* was significantly negatively correlated with *A1* in HL (Supplementary Table 2). These data suggest preferential section of one anti-apoptotic BCL2 family member over another in MZL and HL.

To determine whether individual lymphomas that overexpress *BCLW* simultaneously overexpress other anti-apoptotic BCL2 family members, we selected the top 25% of *BCLW*-overexpressing DLBCL samples, which was validated to highly overexpress *BCLW* (Supplementary Fig. 8), and aligned them with the expression of the other four anti-apoptotic BCL2 family members. We observed 56.8% of the DLBCL samples that overexpressed *BCLW* expressed low (below median) levels of *BCL2* (Fig. 6A and Supplementary Fig. 8). Notably, a third of the DLBCL samples that overexpressed *BCLW* only overexpressed *BCLW* and no other anti-apoptotic BCL2 family members (Fig. 6B).

However, 36.5% of the *BCLW*-overexpressing DLBCL samples also concurrently overexpressed *BCL2*, *BCLX*, *MCL1*, or *A1* (Fig. 6B). Thirty percent of the lymphomas simultaneously overexpressed *BCLW* and two or three other anti-apoptotic BCL2 family members, whereas only one *BCLW*-overexpressing DLBCL also overexpressed the other four family members (Fig. 6B).

We expanded our analysis to evaluate the expression patterns of the other anti-apoptotic BCL2 family members in FL, MZL, MCL, and HL in the top 25% of *BCLW*-overexpressing lymphomas (Fig. 6C-F). In MZL, MCL, and HL, of the lymphomas that overexpressed *BCLW*, 24.3–33.3% only overexpressed *BCLW*, whereas only 12.2% of the FL that overexpressed *BCLW* solely overexpressed *BCLW*. Instead, *BCLW*-overexpressing FL preferentially overexpressed (42.9%) two other anti-apoptotic BCL2 family members (Fig. 6C). We show that two or more anti-apoptotic BCL2 family members are frequently overexpressed together, suggesting that these lymphomas likely rely on more than one for survival. Collectively, our data provide evidence that BCLW is a previously unappreciated contributor to 6 different B-cell lymphomas and that B-cell lymphomas frequently select for the overexpression of BCLW as well as other anti-apoptotic BCL2 family members.

Discussion

Until our recent investigation of Burkitt lymphoma and DLBCL showing that BCLW significantly contributes to these lymphomas (9), BCLW was considered only necessary for spermatogenesis and dispensable for hematopoietic cells (7,8,22,23). Here, we significantly expand upon the lymphomas that select for BCLW overexpression, revealing that both aggressive and indolent lymphomas and non-Hodgkin and Hodgkin lymphomas commonly overexpress BCLW. Our data show BCLW is overexpressed at a frequency that is similar to or greater than, depending on the lymphoma subtype, the quintessential BCL2. Unexpectedly, FL, in which BCL2 overexpression is diagnostic, also overexpressed BCLW at the same frequency as BCL2 overexpression. We also determined a significant inverse association existed between *BCLW* and *BCL2* levels in DLBCL, MZL, and HL, indicating these lymphomas preferentially select for overexpression of one over the other. For HL, *BCLW* was preferentially selected for overexpression over *BCL2*, with samples containing high levels of *BCLW* and low levels of *BCL2*. A fraction of each lymphoma subtype evaluated preferentially selected for *BCLW* overexpression alone. Together, our results indicate that BCLW has a significant role in B-cell lymphomas and is likely as important as BCL2.

Our results also provide an unprecedented evaluation of all five anti-apoptotic BCL2 family members in the six major B-cell lymphoma subtypes. Analyses of the anti-apoptotic BCL2 family members revealed overexpression of more than one family member is a frequent event in all six lymphoma subtypes evaluated. These data suggest a threshold level of expression of BCL2 anti-apoptotic proteins may be needed and once it is reached, overexpression of others is not selected for. However, overall, when mean expression levels in the six lymphomas were compared to normal B-cells, there appeared to be preferential selection for *BCLW*, *BCL2*, and/or *BCLX* overexpression, but not *MCL1* or *A1*. Interestingly, in Burkitt lymphoma, the mean expression of *MCL1* and *A1* was actually

lower than their levels in normal human B-cells, suggesting MCL1 and A1 may not significantly contribute to this lymphoma. However, in individual lymphomas of all subtypes, of those that overexpressed *BCLW*, a similar low frequency of co-overexpression of *BCL2*, *BCLX*, *MCL1*, or *A1* was detected. Our data provide evidence that each B-cell lymphoma subtype is heterogeneous at the anti-apoptotic BCL2 family level, but most have a preference for which family member to overexpress.

Therapies targeted at anti-apoptotic BCL2 family members have entered the clinic. ABT-737 and ABT-263 (navitoclax), which inhibit BCL2, BCLX, and BCLW showed promise in chronic lymphocytic leukemia (CLL) (24). The agents TW-37 and Apogossypolone also target multiple anti-apoptotic BCL2 family members (25). However, ABT-263, TW-37, and Apogossypolone cause acute cytotoxicity from thrombocytopenia (25), which has led to their limited use and the development of compounds only targeting one of the family members. The more selective BCL2 inhibitor, ABT-199 (venetoclax) does not deplete platelets, but causes neutropenia when used with other drugs (26). Targeting BCL2 with a *BCL2* anti-sense DNA oligonucleotide (PNT100) leads to lymphopenia (27). The BCLX-specific inhibitors, A1155463 and A1331852, have been evaluated in non-small cell lung cancer (NSCLC), breast cancer, and T-cell acute lymphoblastic leukemia cell lines (28). MCL1 targeted therapy has shown single-agent killing of multiple myeloma and NSCLC (29). So far, only ABT-199 has been FDA approved for treatment of relapsed or refractory CLL (30). Since we have exposed BCLW as a critical contributor to B-cell lymphomagenesis, BCLW-specific inhibitors will need to be developed and tested, alone and in combination with, other selective BCL2 family inhibitors for future lymphoma treatment.

Despite significant investments into the development of small-molecule therapeutics, many compounds still fail in the clinic due to acquired resistance. Resistance mechanisms to specific anti-apoptotic BCL2 family inhibitors can occur at least, in part, by overexpression of other BCL2 family members. For example, while mantle cell lymphomas are more resistant to ABT-199 when they express increased levels of BCLX and MCL1, knockdown of *MCL1* confers sensitivity to ABT-199 (31). In addition, development of ABT-199 resistance was linked to increased BCLX and MCL1 levels (BCLW not evaluated) in two DLBCL cell lines (32). Additionally, we recently reported that BCLW or BCL2 overexpression conferred resistance to ABT-263 in Burkitt lymphoma cells (9). Therefore, it will be important to monitor other anti-apoptotic BCL2 family members when using therapies that target one or more of them.

Our comprehensive evaluation of BCLW and four other anti-apoptotic BCL2 family members in six different lymphomas reveals the contribution these genes have to B-cell lymphomas by the frequency they are selected for overexpression, individually and with each other. Importantly, *BCLW* overexpression was selected for in all six non-Hodgkin and Hodgkin lymphomas evaluated. Furthermore, each of the lymphomas frequently selected other anti-apoptotic BCL2 family members to overexpress in addition to *BCLW*. These results illustrate the complexity of treating these lymphomas, as they depend on one or more anti-apoptotic BCL2 family members. At a time when inhibitors are actively being developed for use in the clinic to target individual BCL2 family members, our analysis

demonstrates the importance of considering the expression of all anti-apoptotic BCL2 family members for improved targeted therapies and to minimize the occurrence of drug resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Zhijiu Zhong for performing the BCLW IHC in the Translational Pathology Shared Resource core supported by NCI Cancer Center support grant P30CA056036. Support for this study was also provided by NCI R01CA148950 and R21CA184352 (CME) and the Sidney Kimmel Cancer Center.

Funding: Support for this study was provided by NCI R01CA148950 and R21CA184352 (CME), NCI P30CA056036, and the Sidney Kimmel Cancer Center.

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Translational Relevance

With clinical trials on-going for BCL2-specific inhibitors for the treatment of lymphomas and BCLX- and MCL1-specific inhibitors being developed, there is a critical need for increased knowledge of the expression of anti-apoptotic BCL2 family members in lymphomas to identify those most likely to respond to specific inhibitors. Our study, the first large-scale analysis of all anti-apoptotic BCL2 family members in six types of B-cell lymphomas (non-Hodgkin and Hodgkin), reveals B-cell lymphomas typically overexpress more than one. Moreover, BCLW, a previously uncharacterized anti-apoptotic BCL2 family member, was frequently overexpressed. Specific lymphomas preferentially selected for BCLW overexpression over that of BCL2. Notably, BCLW was overexpressed in follicular lymphoma, indicating it likely has a role in this lymphoma. Our results suggest targeting BCLW in B-cell lymphomas may be needed alone or in combination with other specific BCL2 family inhibitors, as targeting just one may lead to dependence on another resulting in therapy resistance.

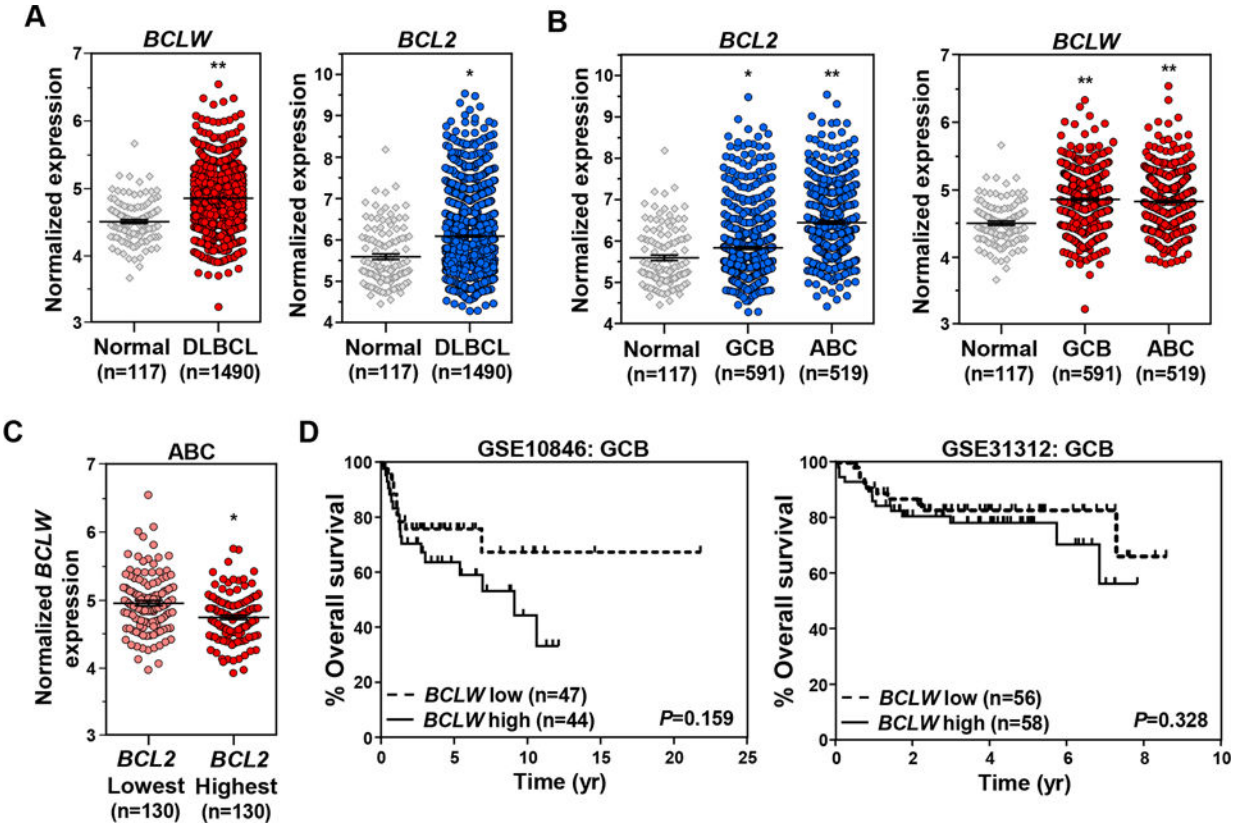

Figure 1. BCLW is overexpressed in GCB and ABC DLBCL

A and B) Gene expression data sets for *BCLW* and *BCL2* mRNA in all DLBCL patient samples evaluated (n=1490; A), or stratified based on GCB (n=591) and ABC (n=519) DLBCL subtypes (B) compared to normal human B-cells (n=117; A and B). C) Association between *BCLW* and *BCL2* mRNA in ABC DLBCL stratified based on the lowest (n=130) and highest (n=130) quartiles of *BCL2* expression. For A, $*P=4.03\times10^{-11}$ and $**P=3.00\times10^{-21}$; B, $*P=1.66\times10^{-3}$ and $**P<4.78\times10^{-18}$; C, $*P=1.34\times10^{-5}$; two-tailed t-tests. D) Patient samples of GCB DLBCL with low *BCL2* from GSE10846 and GSE31312 data sets were each stratified based on median expression of *BCLW* into two groups, *BCLW* low and *BCLW* high. Kaplan-Meier overall survival analyses were performed and compared using log-rank tests.

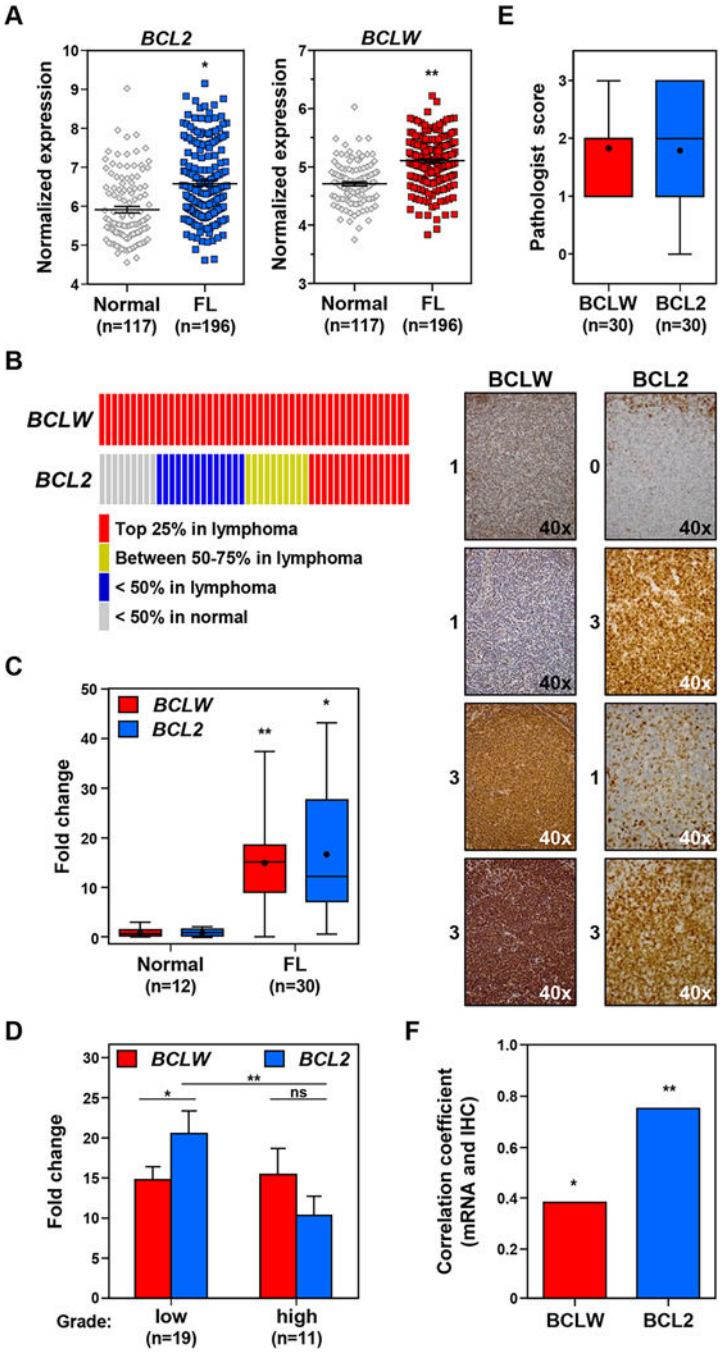

Figure 2. Overexpression of BCLW is selected for in follicular lymphoma

A) Gene expression data for *BCL2* and *BCLW* mRNA in FL (n=196) compared to normal human B-cells (n=117). *P=5.64×10^{-10} and **P=1.11×10^{-16}; two-tailed t-tests. B) Association between *BCLW* and *BCL2* mRNA in FL stratified based on the highest quartile (n=49) of *BCLW* expression. Each line represents one sample. C and D) qRT-PCR analysis for *BCLW* and *BCL2*, in triplicate, from FL patient samples (n=30) compared with normal lymph nodes (n=12) in C (box and whisker plot), or stratified based on grade (low, n=19; high, n=11) in D. For C, *P=5.91×10^{-5} and **P=7.21×10^{-7}; D, *P=0.044 and **P=0.023;

two-tailed *t*-tests. E) IHC analysis for BCLW and BCL2 protein on FL patient samples (n=30). Box and whisker plot of pathologist scores and representative images with scores of BCLW and BCL2 from the same tumors are shown. F) Pearson correlation coefficients for BCLW and BCL2 comparing mRNA fold-change to IHC scores, $*P$=0.039 and $**P=1.73\times10^{-6}$, two-tailed *t*-tests.

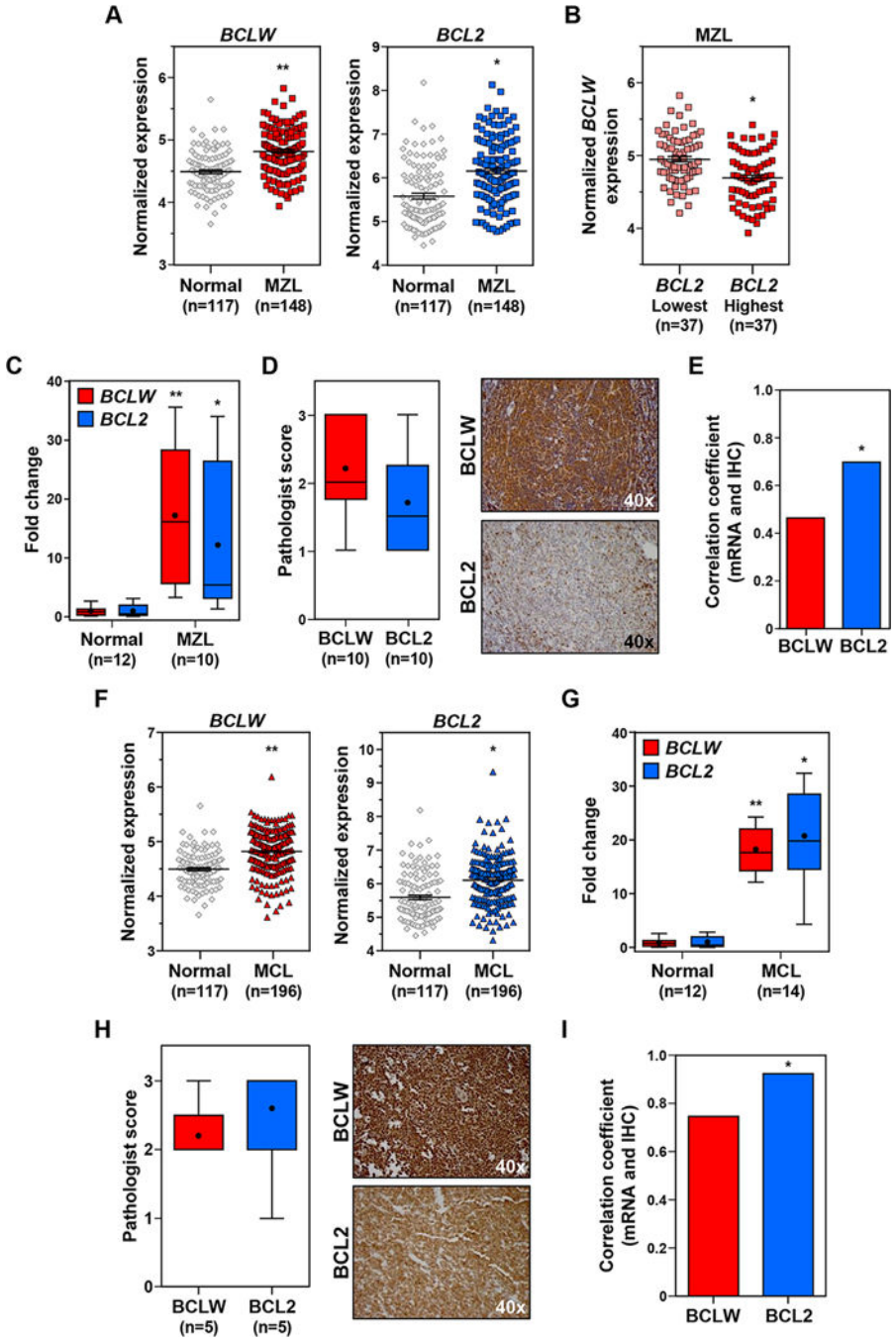

Figure 3. Marginal zone and mantle cell lymphomas overexpress BCLW

A and F) Gene expression data for *BCLW* and *BCL2* mRNA in MZL (n=148, A) and MCL (n=196, F) compared to normal human B-cells (n=117). For A, $*P=7.38\times10^{-10}$ and $**P=1.29\times10^{-13}$; F, $*P=2.94\times10^{-9}$ and $**P=1.06\times10^{-13}$; two-tailed t-tests. B) Association between *BCLW* and *BCL2* mRNA in MZL stratified based on the lowest (n=37) and highest (n=37) quartiles of *BCL2* expression; $*P=5.13\times10^{-5}$; two-tailed t-tests. C and G) qRT-PCR analysis (box and whisker plot) for *BCLW* and *BCL2*, in triplicate, from nodal MZL (n=10, C) and MCL (n=14, G) patient samples compared with normal lymph nodes (n=12). For C,

$*P=5.95\times10^{-3}$ and $**P=8.03\times10^{-5}$; G, $*P=9.28\times10^{-9}$ and $**P=2.01\times10^{-12}$; two-tailed *t*-tests. D and H) IHC analysis for BCLW and BCL2 protein on nodal MZL ($n=10$, D) and MCL patient samples ($n=5$, H). Box and whisker plots of pathologist scores and representative images of BCLW and BCL2 from the same tumors are shown. E and I) Pearson correlation coefficients for BCLW and BCL2 comparing mRNA fold-change to IHC scores (E, $*P=0.024$; I $*P=0.027$, two-tailed *t*-tests).

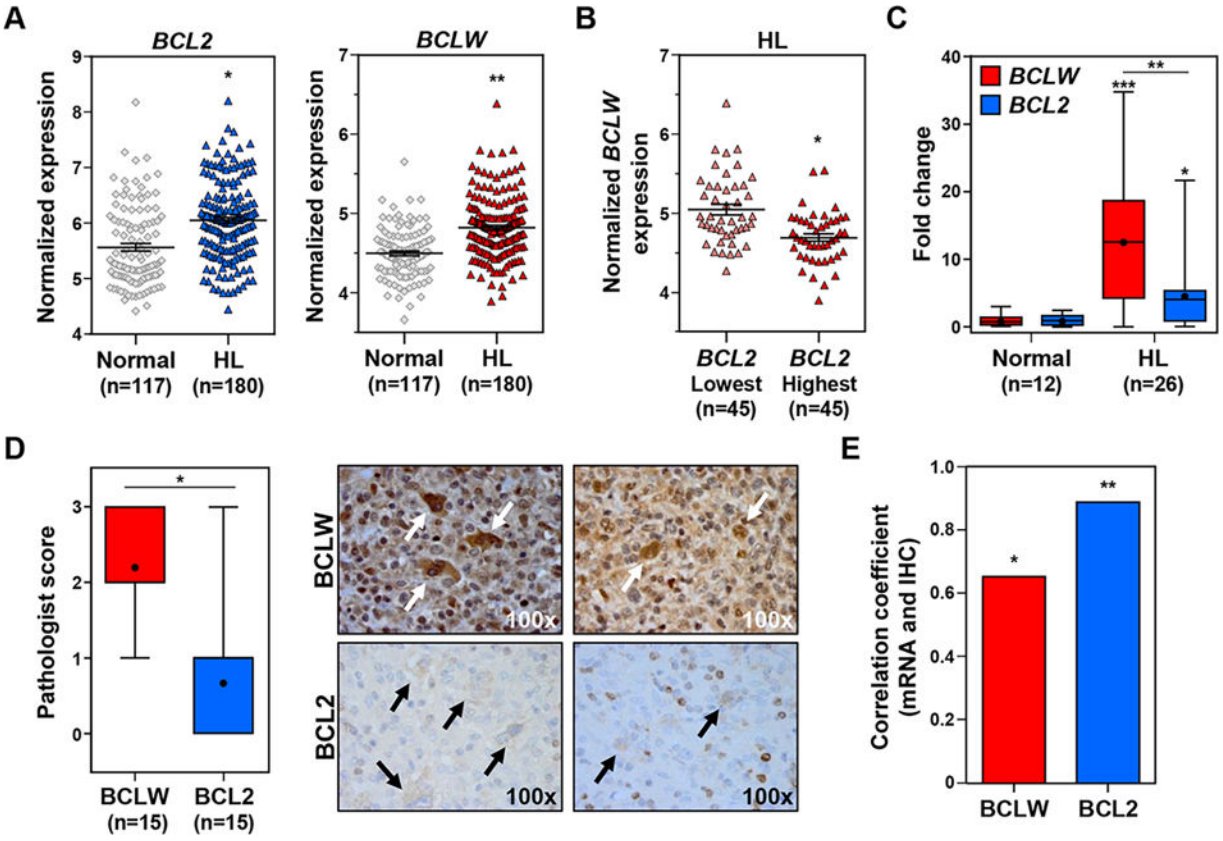

Figure 4. BCLW is preferentially overexpressed in Hodgkin lymphoma

A) Gene expression data for *BCL2* and *BCLW* mRNA in HL (n=180) compared to normal human B-cells (n=117). B) Association between *BCLW* and *BCL2* mRNA in HL stratified based on the lowest (n=45) and highest (n=45) quartiles of *BCL2* expression. For A, $*P=2.27\times10^{-8}$ and $**P=5.25\times10^{-14}$; B, $*P=4.18\times10^{-5}$; two-tailed t-tests. C) Box and whisker plot of qRT-PCR analysis for *BCLW* and *BCL2*, in triplicate, from HL patient samples (n=26) compared with normal lymph nodes (n=12). $*P=0.013$, $**P=2.54\times10^{-4}$, and $***P=6.26\times10^{-5}$; two-tailed t-tests. D) IHC analysis for BCLW and BCL2 protein on HL patient samples (n=15). Box and whisker plots of pathologist scores and representative images of BCLW and BCL2 from the same tumors are shown; arrows indicate HL cells. $*P=1.43\times10^{-5}$; two-tailed t-test. E) Pearson correlation coefficients for BCLW and BCL2 comparing mRNA fold-change to IHC scores, $*P=8.49\times10^{-3}$ and $**P=1.10\times10^{-5}$, two-tailed t-tests

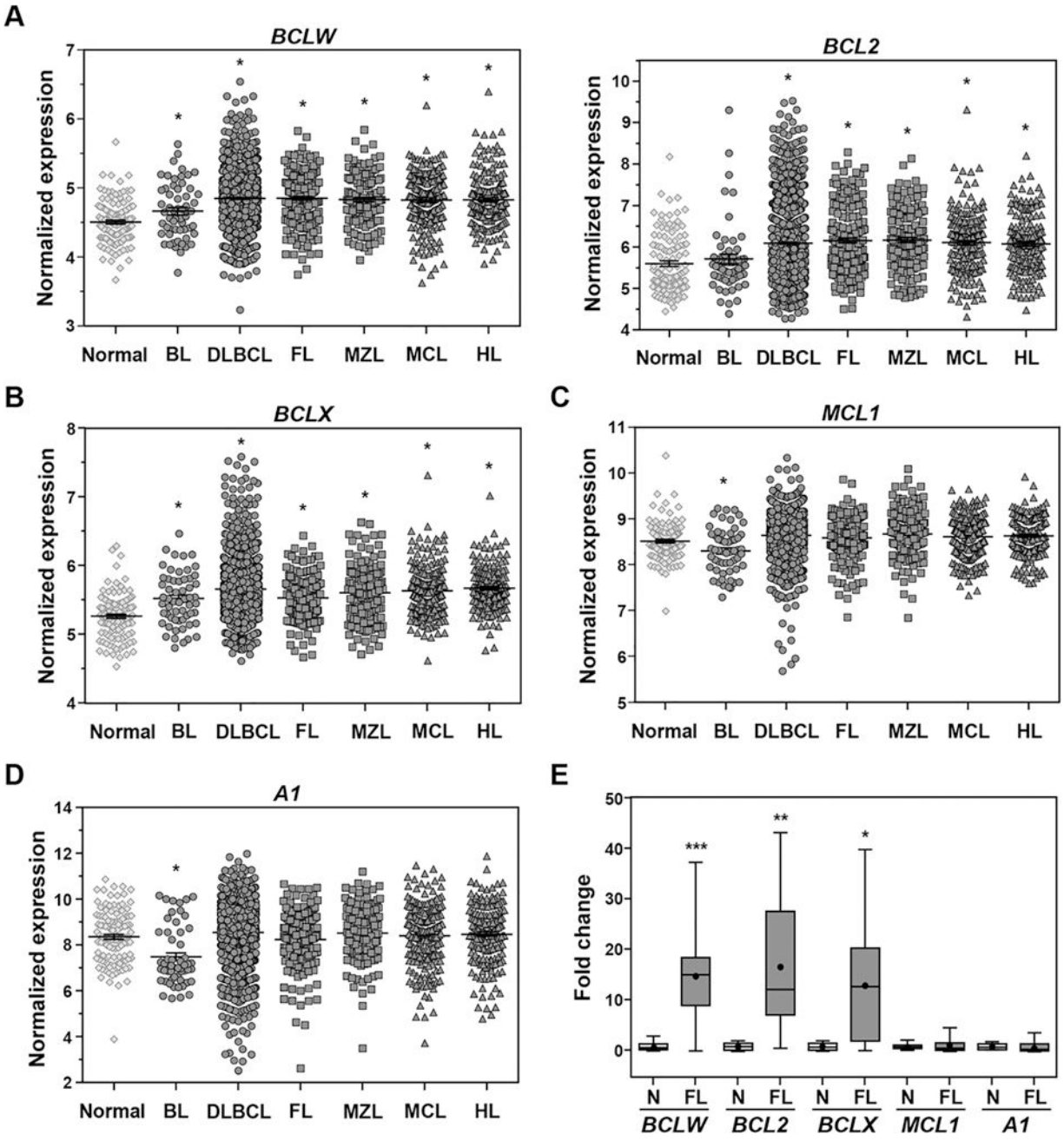

Figure 5. Expression of anti-apoptotic BCL2 family members in different B-cell lymphomas

A-D) Gene expression data for *BCLW* and *BCL2* (A), *BCLX* (B), *MCL1* (C), and *A1* (D) mRNA in the six different lymphomas indicated (BL, Burkitt lymphoma). The data in A is summarized from A in Figures 1–4 and 3F. $*P<1.0\times10^{-3}$ is significant except for BL ($*P<0.01$ due to small sample size), two-tailed *t*-tests. E) Box and whisker plot of qRT-PCR analysis for *BCLW*, *BCL2*, *BCLX*, *MCL1*, and *A1* mRNA, in triplicate, from FL patient samples ($n=30$) compared with normal lymph nodes (N; $n=12$). The data in E for *BCLW* and *BCL2* is the same as Figure 2C, but included here for comparison. $*P=3.77\times10^{-4}$, $**P=5.91\times10^{-5}$, and $***P=7.21\times10^{-7}$; two-tailed *t*-tests.

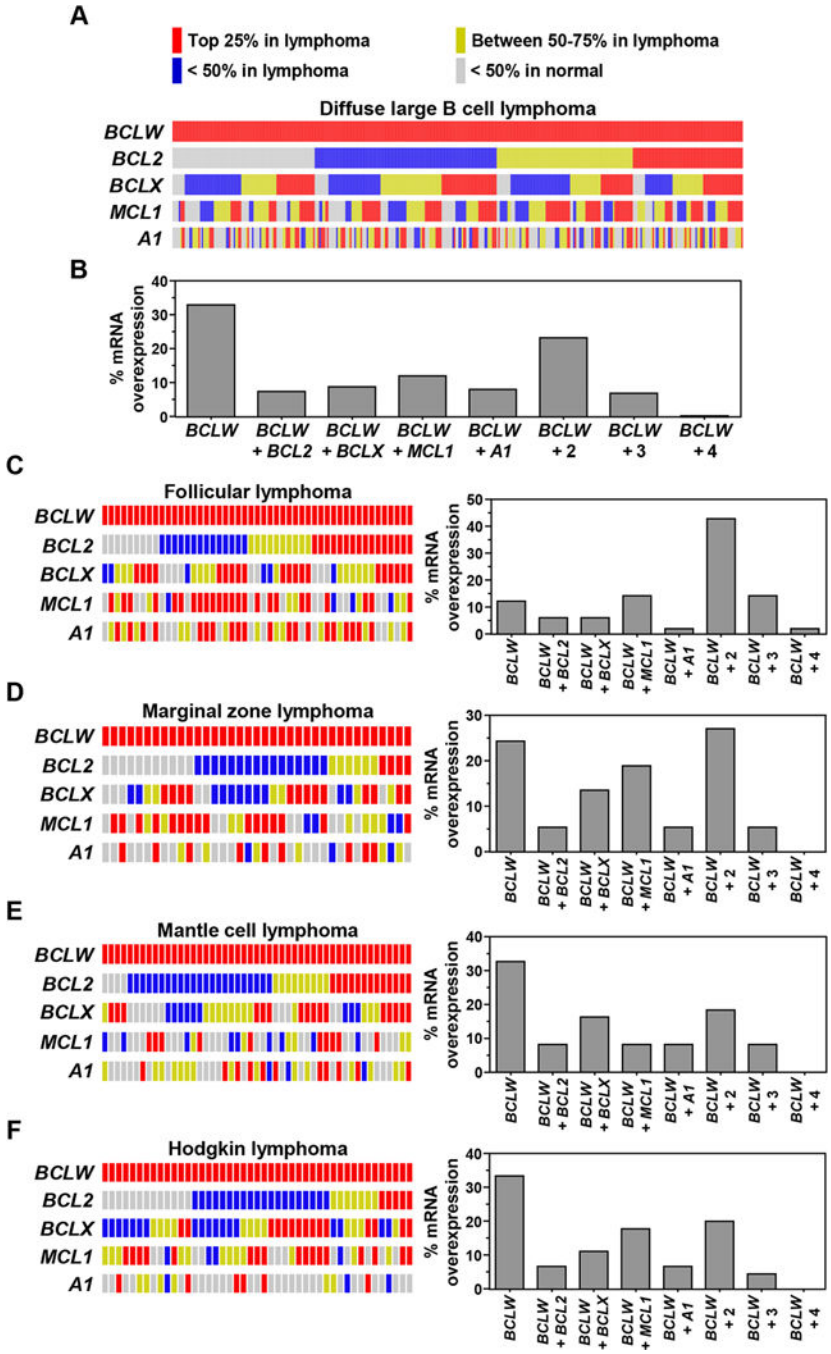

Figure 6. Non-Hodgkin and Hodgkin lymphomas preferentially overexpress select anti-apoptotic BCL2 family members

A) Gene expression data showing association between *BCLW* and *BCL2*, *BCLX*, *MCL1*, and *A1* mRNA in DLBCL stratified based on the top 25% of *BCLW* expression. Each line represents one sample. B) Summarized results of A showing the percentage of samples with overexpression of the indicated mRNA within the top 25% of *BCLW* expression in DLBCL. The +2, +3, and +4 indicate the number of additional anti-apopototic BCL2 family members that were also overexpressed. C-F, left) Gene expression data showing association between *BCLW* and *BCL2*, *BCLX*, *MCL1*, and *A1* mRNA in FL (C), MZL (D), MCL (E), and HL

(F) stratified based on the top 25% of *BCLW* expression. Each line represents one sample. C-F, right) Summarized results of left panels showing the percentage of samples with overexpression of the indicated mRNA within the top 25% of *BCLW* expression.