LYMPHOID NEOPLASIA

BCL2 mutations are associated with increased risk of transformation and shortened survival in follicular lymphoma

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Key Points

- *BCL2* mutations in FL correlate with activation-induced cytidine deaminase expression and frequently alter the amino acid sequence of the protein.
- Mutations in the *BCL2* coding sequence at diagnosis are associated with shortened time to transformation and earlier death due to lymphoma.

Follicular lymphoma (FL), an indolent neoplasm caused by a t(14;18) chromosomal translocation that juxtaposes the *BCL2* gene and immunoglobulin locus, has a variable clinical course and frequently undergoes transformation to an aggressive lymphoma. Although *BCL2* mutations have been previously described, their relationship to FL progression remains unclear. In this study, we evaluated the frequency and nature of *BCL2* mutations in 2 independent cohorts of grade 1 and 2 FLs, along with the correlation between *BCL2* mutations, transformation risk, and survival. The prevalence of *BCL2* coding sequence mutations was 12% in FL at diagnosis and 53% at transformation ($P < .0001$). The presence of these *BCL2* mutations at diagnosis correlated with an increased risk of transformation (hazard ratio 3.6; 95% CI, 2.0-6.2; $P < .0001$) and increased risk of death due to lymphoma (median survival of 9.5 years with *BCL2* mutations vs 20.4 years without; $P = .012$). In a multivariate analysis, *BCL2* mutations and high FL international prognostic index were independent risk factors for transformation and death due to lymphoma. Some mutant Bcl-2 proteins exhibited enhanced antiapoptotic capacity in vitro. Accordingly, *BCL2* mutations can affect antiapoptotic Bcl-2 function, are associated with increased activation-induced cytidine deaminase expression, and correlate with increased risk of transformation and death due to lymphoma. (*Blood*. 2015;125(4):658-667)

Introduction

Follicular lymphoma (FL) has a highly variable clinical course.[1-3] Although some patients do well for decades, often with limited therapy, at some point 30% to 50% of patients experience histologic transformation to a more aggressive lymphoma, usually diffuse large B-cell lymphoma (DLBCL).[4-11] This transformation, which is thought to reflect the acquisition of new genetic abnormalities leading to further genomic instability,[12-16] has generally been associated with a poor clinical outcome.[17] Retrospective analyses from the prerituximab era have reported a median survival of only 1 to 2 years after transformation,[18,19] although a recent prospective observational study suggests somewhat better survival after transformation in the rituximab era.[20] At the present time, the FL international prognostic index (FLIPI), which integrates patient characteristics at diagnosis, is the gold standard for predicting FL clinical outcome.[21,22] There is, however, considerable interest in identifying characteristics of the FL cells themselves that might also impact prognosis.[22,23]

The *BCL2* gene is critical for FL pathogenesis.[24,25] Originally identified because of its translocation to the immunoglobulin heavy chain (*IGH*) locus as a part of the t(14;18) translocation that typifies FL, *BCL2* encodes a protein that inhibits apoptosis.[26,27] In particular, Bcl-2 and related antiapoptotic proteins diminish apoptosis by binding and neutralizing activated proapoptotic Bcl-2 family members, including the mitochondrial outer membrane permeabilizers Bax and Bak, as well as the intracellular stress sensors Bim and Puma that activate Bax and Bak.[27-29] Because the *IGH* locus is activated in B cells, the translocated *BCL2* gene is overexpressed in FL cells[25] and enhances their survival.[30]

Activation-induced cytidine deaminase (AID), which is highly expressed in germinal center B cells, introduces mutations into the variable and switch regions of B-cell receptor genes during the physiological process of secondary antibody diversification.[31] After AID deaminates cytidine to uracil, the resulting uracil-guanine

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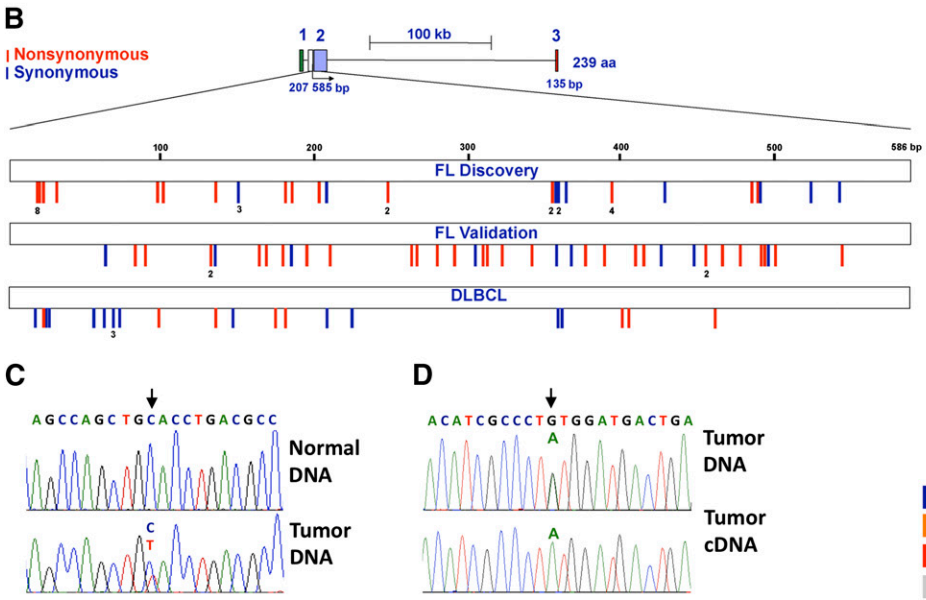
Number of Samples	Number of Nonsynonymous Mutations [1]	Number of Synonymous Mutations [1]	Mutations per Kb [2]
Normal (n=300)	0	0	-
FL Discovery (n=38)	26 (68.5)	12 (31.5)	1.71
FL Validation (n=128)	30 (76.9)	9 (23.1)	0.52
DLBCL (n=39)	8 (36.5)	14 (63.5)	0.96
DLBCL *Ig*-BCL2 (n=23)	8 (42)	11 (58)	1.41
Post FL (n=117)	1 (50)	1 (50)	0.03
CLL (n=90)*	0	2	0.07
ALL (n=36)	0	0	-

[1]numbers in parentheses represent percentage of total substitutions
[2]number of mutations divided by the length of exon 2
*40 without *IGH* rearranged and 50 with *IGH* rearranged



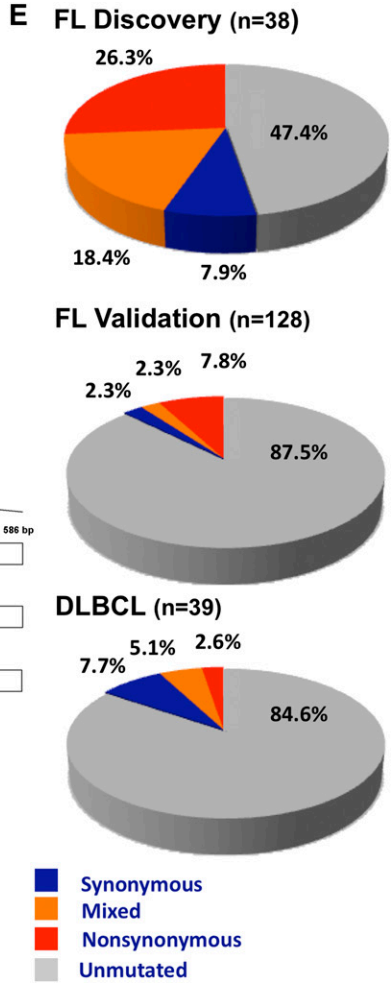


Figure 1. Distribution of *BCL2* somatic mutations in FL and non-Hodgkin lymphoma subtypes. (A) Summary of somatic sequence mutations for *BCL2* in FL and other non-Hodgkin lymphoma subtypes analyzed by Sanger sequencing in normal samples, a discovery (prevalence) cohort of 38 FLs, a validation cohort of 128 FLs collected exclusively at diagnosis, and cohorts of 117 post-FLs, 39 DLBCLs, 90 CLLs (50 with *IGH* rearrangement and 40 without), and 36 ALLs. (B) Schematic diagram of the *BCL2* gene (top) and somatic mutation distribution along the *BCL2* gene in the FL discovery cohort (n = 38), FL validation cohort (n = 128), and DLBCL cohort (n = 39). Exons 1, 2, and 3 are coded in green, purple, and red, respectively. Blue and red bars indicate synonymous and nonsynonymous mutations, respectively. (C-D) Sequencing chromatograms of representative mutated FL samples (lymph node) and paired normal (peripheral blood) DNA (C), or FL DNA and corresponding cDNA (D). Arrows point to the positions of nucleotide change, with the variant nucleotide labeled in red. (E) Overall frequency of *BCL2* somatic mutations in FL and DLBCL. Within each group, samples without mutation are depicted in gray, samples with only synonymous mutations in blue, samples with mixed mutations in orange, and samples with nonsynonymous mutations in red.

mismatches are replicated to produce a C→T transition, a hallmark of AID activity, or removed by uracil DNA glycosylase to create an abasic site that is recognized by nucleases and error prone polymerases, leading to a variety of other mutations or DNA double-strand breaks.[32,33] In addition to its role in antibody diversification, AID is postulated to play a critical role in lymphomagenesis by mutating genes outside the immunoglobulin locus,[32,34] including the proto-oncogenes *BCL6*, *PIM1*, *RhoH/TTF*, *PAX5*, and *MYC*.[35,36] Moreover, AID contributes to genetic instability, including the acquisition of chromosomal abnormalities such as *IGH* translocations.[37,38] Even though AID levels vary substantially among different FLs,[34] the implications of this variation have been unclear.

At the time *BCL2* was cloned, single nucleotide variants (SNVs) were observed in human lymphoma cell lines[39] and a handful of clinical lymphomas[40] compared with normal tissues. Because these *BCL2* variants, like wild-type (WT) *BCL2*, inhibited cell death[41] and facilitated lymphomagenesis,[42] it was assumed that *BCL2* mutations do not affect Bcl-2 protein function. Consistent with this possibility, previous studies showed that the majority of *BCL2* mutations in DLBCL were silent[43,44] and had no effect on survival.[44]

More recently, large-scale genomic studies have also identified frequent *BCL2* mutations in FL.[16,45] Whether these *BCL2* mutations have a biological impact has been unclear. Our previous studies demonstrated that some mutant Bcl-2 proteins found in lymphoma cell lines exhibit enhanced affinity for proapoptotic Bcl-2 family members and increased the ability to protect from death stimuli.[46,47] Accordingly, the present studies were designed to assess the occurrence, nature, and potential clinical importance of mutations in the *BCL2* coding sequence in FL.

Methods

Patients

Patients with grades 1 and 2 FL who provided written consent for enrollment in the actively maintained Mayo Clinic Lymphoma Database were studied. After approval by the Mayo Clinic Institutional Review Board (Rochester, MN), slides from initial diagnostic biopsies and transformation were subjected to pathology review applying World Health Organization criteria.

Table 1. Characteristics of patients in FL discovery and validation cohorts

Characteristic	Discovery cohort (n = 38)	Validation cohort (n = 128)
Age (median, range)	63 (27-84)	64 (26-91)
Sex, male n (%)	20 (51%)	55 (43%)
Stage		
1	16 (44%)	18 (14%)
2	16 (43.25%)	27 (21%)
3	6 (16.25%)	23 (18%)
4	0 (0%)	61 (47%)
B symptoms (present)	7	13
LDH (elevated)	3	11
FLIPI		
0-1	14 (37%)	53 (41%)
2	11 (29%)	53 (41%)
3-4	13 (34%)	22 (17%)
Unknown	0 (0%)	1 (1%)
Initial therapy	**Therapy prior to sample**	**Initial therapy**
Observation	23 (60.5%)	59 (46%)
Chemotherapy	8 (21%)	36 (28%)
Chemotherapy plus rituximab	2 (5.3%)	3 (2%)
Rituximab	0 (0%)	3 (2%)
RT	0 (0%)	16 (12%)
RT plus chemotherapy	5 (13.2)	2 (2%)
Surgical resection	0 (0%)	10 (8%)

RT, radiation therapy; LDH, lactate dehydrogenase.

Patients with grade 3 FL were excluded. Three distinct cohorts were studied. Research was conducted in accordance with the Declaration of Helsinki.

Discovery cohort. The discovery cohort consisted of 38 FL patients with frozen tissue samples stored in the Mayo Clinic Lymphoma Tissue Bank at diagnosis (n = 20) or during the course of the disease (n = 18) and available clinical data, including outcome (median follow-up, 10.5 years). Of the 18 patients biopsied during the course of the disease, 16 had received prior therapy with various regimens. The discovery cohort was used to explore the frequency of *BCL2* mutations, as well as the relationship between *BCL2* mutation status and clinical outcome.

Validation cohort. The validation cohort consisted of 128 patients with newly diagnosed FL seen at the Mayo Clinic between May 1975 and July 2005 who were included in an actively maintained database, and had (1) formalin-fixed paraffin embedded (FFPE) samples available from biopsy at initial diagnosis and (2) tissue available from the time of transformation if transformation occurred. Visual scoring of hematoxylin and eosin slides from the same block by an experienced hematopathologist indicated that samples contained a median of 70% (range, 30% to 90%) neoplastic cells. The validation cohort was used to assess the frequency of mutations in the coding regions of *BCL2* at diagnosis and transformation, as well as the relationship between *BCL2* mutations at diagnosis and clinical outcome in FL patients with uniformly recorded clinical characteristics, long-term follow-up, and known outcome.

Paired FL/germline control. Paired samples of peripheral blood DNA and lymph node biopsy from 11 FL patients at diagnosis were examined to evaluate the possibility that FL SNVs represent germline variants rather than somatic mutations. Because patients in this group had a relatively short follow-up (5.5 years), samples were used for tumor/germline comparison but were not included in outcome analysis.

Other B-cell lymphomas and normal controls. To assess the frequency of *BCL2* mutations in other lymphoma subtypes, DNA was isolated from 39 DLBCLs, 117 mucosa-associated, marginal zone, and lymphoplasmacytic ("postfollicular") lymphomas, 36 acute lymphocytic leukemias (ALLs), and 90 chronic lymphocytic leukemias (CLLs), 40 without and 50 with *IGH* variable region somatic mutations. DNA from 300 normal individuals (100 Caucasians, 100 African American, and 100 Han Chinese) was purchased from the Coriell Institute (Camden, NJ).

Isolation of genomic DNA, sequencing, and identification of tumor-specific mutations

DNA was isolated using a QIAamp DNA Kit (Qiagen). For FFPE FL samples, an Illumina Infinium HD FFPE Restore Kit (Illumina) and QIAamp DNA FFPE Kit (Qiagen) were used. Polymerase chain reaction (PCR) was performed using primers (see supplemental Table 1, available on the *Blood* Web site) for exons 1 (discovery cohort only), 2, and 3 of *BCL2* (NG_009361.1). PCR products were subjected to Sanger sequencing on both strands using ABI PRISM BigDye Terminator technology at the Mayo Clinic DNA Sequencing Core. All SNVs were detected in both 3′ and 5′ directions and confirmed in a separate sequencing reaction using independently amplified DNA. Mixing experiments demonstrated that this approach readily detects variants that are present in ≥10% of DNA molecules. Candidate somatic variants (tumor-specific) were identified after removing known polymorphisms present in NCBI dbSNP database, HapMap, and in our set of normal samples. The absence of the identified mutations in the COSMIC database and in the Cancer Gene Census database was verified. A similar approach was likewise used to sequence the exons of *PIM1* using primers shown in supplemental Table 1.

RNA isolation

RNA was extracted from frozen FL samples using an RNeasy Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized with Superscript III, amplified with Promega master mix and primers (supplemental Table 1), and sequenced as described above.

AID immunostaining and messenger RNA (mRNA) detection

FL samples were stained with murine monoclonal anti-AID primary antibody (cat #39-2500; Life Technologies) and peroxidase-labeled secondary antibody. Samples were scored based on the percentage of tumor cells expressing AID, with <20%, 21% to 40%, 41% to 60%, 61% to 80%, and >80% being scored 1, 2, 3, 4, and 5, respectively, by a lymphoma pathologist blinded to outcome data. AID mRNA was assessed by semi-quantitative reverse transcription PCR (RT-PCR) using primers (5′-AGGCAAGAAG-ACACTCTGGACACC-3′ and 5′-TCAAAGTCCCAAAGTACGAAATGC-3′) with β-actin as a positive control.

Statistical analysis

Risk of transformation over time and time to transformation (TTT) were estimated as time from diagnosis to histologically proven transformation using Kaplan-Meier methods[48]; patients without transformation at the time of last follow-up or death were censored. The FL-specific survival was estimated as the time from diagnosis to lymphoma-associated death, or last follow-up, using Kaplan-Meier methods. Patients alive at last follow-up or with death not lymphoma related were censored. The log-rank test and Fisher's exact test were used to test for association between variables as appropriate. The Cox proportional hazards model was used to evaluate the impact of prognostic variables on TTT and lymphoma-specific survival, and to adjust for other known prognostic variables in FLIPI (0 to 2 vs 3 to 4).[49] All *P* values were two-sided and $P < .05$ was considered to be statistically significant. All analyses were performed using JMP 9.0 statistical discovery software by SAS.

Results

BCL2 mutation frequency in FL

Based on recent studies reporting frequent *BCL2* mutations in FL,[16,44,45] as well as our previous studies suggesting that certain Bcl-2 sequence variants found in lymphoma cell lines exhibit enhanced antiapoptotic properties,[46,47] we examined the occurrence and significance of mutations in the *BCL2* coding sequence in FL. In an initial prevalence study, *BCL2* exons 1, 2, and 3 were sequenced in cryopreserved FL specimens from 38 FL patients (termed the "discovery"

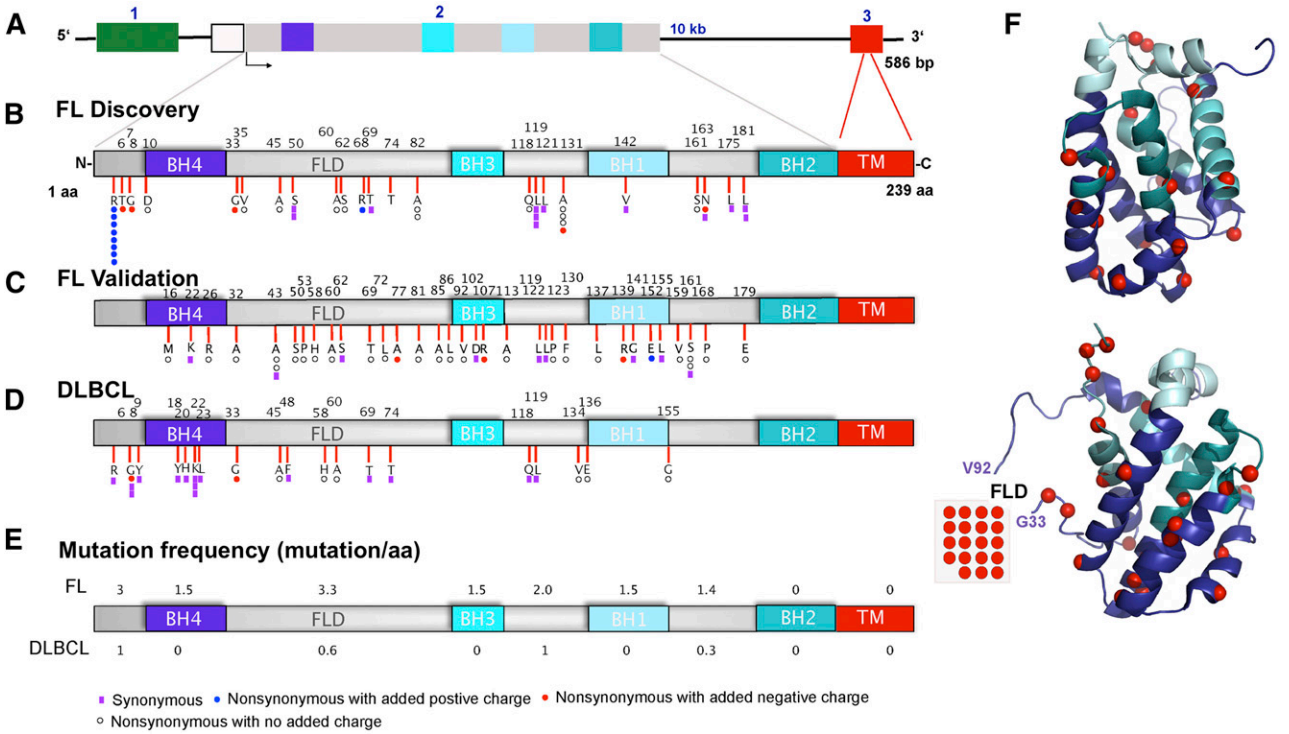


Figure 2. Distribution of mutations in the Bcl-2 protein. (A) Schematic diagram of the *BCL2* gene. Exons 1, 2, and 3 are color-coded in green, gray, and red, respectively. (B-D) Linear diagram of Bcl-2 protein showing Bcl-2 homology domains and distribution of amino acid alterations in (B) FL discovery (n = 38), (C) FL validation (n = 128), and (D) DLBCL (n = 39) cohorts. Color-coded symbols depict distinct types of alterations, with purple for synonymous, white for nonsynonymous with no charge introduction, red for nonsynonymous with introduction of a negative charge, and blue for nonsynonymous with introduction of a positive charge. (E) Overall *BCL2* coding mutation frequency normalized for an interval of 10 amino acids in FL and DLBCL cohorts. (F) Two different projections of Bcl-2 are presented showing coding somatic mutations (red spheres) in the 2 FL cohorts mapped together onto the Bcl-2 3D structure (PDB 2O21). BH, Bcl-2 homology domain; FLD, flexible loop domain; TM, transmembrane domain.

cohort) who had a median age of 64 years, median follow-up of 10.5 years, and median survival of 13.2 years. DNA from 300 normal individuals served as a control. We identified lymphoma-specific variants (supplemental Table 3) by removing polymorphisms present in the control samples, NCBI dbSNP and HapMap.

A total of 38 SNVs were observed in 20 of the 38 discovery cohort samples (Figure 1A; supplemental Table 3). These SNVs were located exclusively within exon 2 and not within the silent exon 1 or within exon 3 that encodes the Bcl-2 transmembrane domain (Figure 1B). To distinguish between rare germline variants and somatic mutations, we sequenced paired DNA samples from peripheral blood mononuclear cells and lymph nodes of 11 FL patients. As illustrated in Figure 1C for 1 case, 8 of these 11 FLs had SNVs in *BCL2* exon 2 that were not present in the corresponding normal cells, indicating that the *BCL2* SNVs represent somatic mutations.

To assess whether *BCL2* mutations occurred in the translocated alleles, we sequenced cDNA prepared from discovery cohort samples with *BCL2* mutations. The mutated message was vastly more abundant than WT in all 20 samples containing *BCL2* mutations (Figure 1D), suggesting that the translocated allele is mutated.

To provide a basis for further examination of *BCL2* mutations, we also sequenced the *PIM1* gene, which is thought (like *BCL2*) to be a target of AID-induced mutagenesis. In the discovery cohort, only 5 of the 39 samples (3 of the 20 *BCL2*-mutant samples) harbored *PIM1* mutations.

The discovery cohort had 1.71×10^{-3} mutations/bp in *BCL2* exon 2, a frequency that approaches the *IGH* mutation rate (5.8×10^{-3} mutations/bp) during B-cell differentiation (Figure 1A).[44,50] The mutation rate was much lower in other B-cell neoplasms, with *BCL2* SNVs in 2 of 117 mucosa-associated, marginal zone, and lymphoplasmacytic ("postfollicular") lymphomas, 0 of 36 ALLs and 2 of 90 CLLs (Figure 1A). Moreover, *BCL2* was mutated twice as often in FL as in DLBCL (Figure 1A-B,E; supplemental Table 3), consistent with earlier results.[44,50] Of the 39 DLBCLs examined, 23 were positive for *Ig-BCL2* rearrangements involving the major breakpoint region (21) or minor cluster region (2), respectively. Nineteen *BCL2* mutations occurred in *BCL2*-rearranged cases and 3 mutations occurred in nonrearranged cases (supplemental Table 3).

Presence of *BCL2* mutations in FL at diagnosis

The discovery cohort contained samples collected at diagnosis and at relapse after chemotherapy. To rule out the possibility that *BCL2* mutations were chemotherapy induced, as well as provide an opportunity for analyzing the relationship between *BCL2* mutations at diagnosis and outcome, *BCL2* sequencing was performed on a second, independent cohort collected exclusively at diagnosis. This validation cohort consisted of 128 patients with grades 1 or 2 FL and a median follow-up of 8.4 years (range, 0 to 30). Patient characteristics and survival are summarized in Table 1 and supplemental Figure 1. Fifty-eight (44%) of these patients had histologic evidence of transformation during follow-up, with a median TTT of 14 years, consistent with earlier findings.[6,10,11] Sequencing revealed a total of 39 *BCL2* mutations in 16 specimens at diagnosis in this cohort (Figure 1A-B).

Nature and patterns of *BCL2* mutations in FL

Further analysis indicated that 26 of 38 mutations in the discovery cohort and 30 of 39 mutations in the validation cohort were amino acid altering (nonsynonymous; Figure 1B,E), providing an overall ratio of 2.7:1 for nonsynonymous vs synonymous mutations in FL.

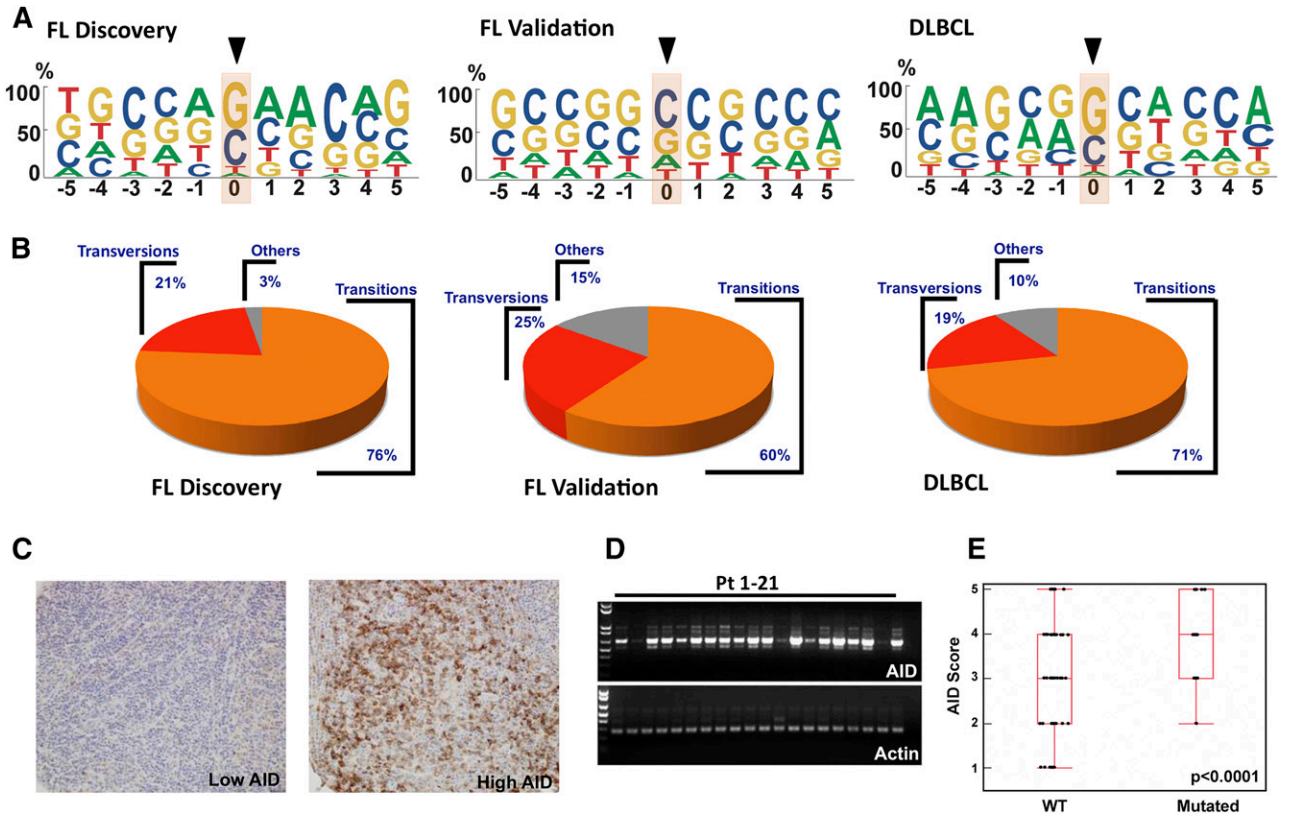


Figure 3. C/G predominance of *BCL2* mutations and AID expression in FL. (A) Depiction of bp preference of mutations in the FL discovery (left), FL validation (middle), and DLBCL (right) cohorts. The size of letters corresponds to the frequency with which that nucleotide is mutated in the *BCL2* coding strand. (B) Transition and transversion load in FL discovery, FL validation, and DLBCL cohorts. Within each group, transitions are depicted in orange, transversions in red, and other changes in gray. (C) Images of FL validation cohort lymph node biopsies from diagnosis showing low (left) and high (right) staining after reaction with anti-AID primary antibody and peroxidase-labeled secondary antibodies. (D) RT-PCR was performed in 21 FL patient samples to detect AID expression differences at the mRNA level. Actin was used as an RNA loading control. (E) AID score (calculated as described in "Methods") from 128 pretreatment FL validation cohort diagnostic samples depicted as a function of *BCL2* mutation status ($P < .0001$).

In contrast, the nonsynonymous:synonymous ratio was 0.57:1 in DLBCL (Figure 1E), in agreement with recent reports.[43,44] Furthermore, some FLs had multiple nonsynonymous mutations (n = 2 in 6 samples, n = 3 in 2 samples, and n = 4, 5, or 10 in 1 sample each). Importantly, 90% of FL synonymous mutations occurred in combination with nonsynonymous SNVs (supplemental Table 3). This high frequency of nonsynonymous mutations, along with the absence of truncating mutations, raised the possibility that *BCL2* mutations might confer an advantage to FL cells.

As indicated in Figure 2A-C, nonsynonymous mutations in FL were spread along the protein, including the *N*-terminus (10 of 77 mutations), flexible loop domain (FLD) (20 of 77), and conserved BH4, BH3, and BH1 domains (31 of 77). The distribution of mutations in DLBCL is shown in Figure 2D for comparison. In FL, the highest mutation frequency (defined as mutation load per 10 bp) occurred in the unstructured FLD (Figure 2E-F and supplemental Figure 2), which was previously implicated in binding p53 and c-myc as well as regulating the affinity of Bcl-2 for proapoptotic Bcl-2 family members.[51-53]

Some 95% of SNVs in the discovery cohort and 71% in the validation cohort occurred at Gs and Cs (Figure 3A-B). Moreover, 76% and 60% of mutations in the discovery and validation cohorts, respectively, were transitions (C to T or G to A) (Figure 3B). This predilection for Gs and Cs, as well as a high transition/transversion ratio, has been observed during AID-induced somatic hypermutation in normal B cells,[31] suggesting that AID might be responsible for the observed *BCL2* mutations. Consistent with this possibility, ~40% of FL SNVs occurred at preferred AID target sequences (GCT/A or A/TGC) (Figure 3A and supplemental Table 4), even though AID can also mutate cytidines in other sequence contexts. Moreover, AID sites were not randomly mutated. Instead, mutations were only observed in exon 2, even though 39 preferred AID sites were present in exons 1 and 3 compared with 12 in exon 2 (supplemental Table 4).

Relationship between AID expression and *BCL2* mutations in FL

When AID expression in the FL validation cohort at diagnosis was examined, immunohistochemistry demonstrated substantial inter-sample variability (Figure 3C). Similar variability was observed when AID was assessed by RT-PCR (Figure 3D). Importantly, AID expression was higher in samples with *BCL2* mutations than in those without (Figure 3E) ($P < .0001$).

Increased *BCL2* mutations at transformation

The presence of the mutagenic AID enzyme in many FLs raised the possibility that *BCL2* mutations might continue to accumulate during the course of disease. To assess this possibility, we sequenced *BCL2* in 31 available FL pairs, the first collected at diagnosis (part of the validation cohort) and the second at median of 4.5 (range, 0.5 to 14.5) years later at transformation. In these 31 pairs, the prevalence of *BCL2* mutations was 16% at diagnosis and 65% at transformation (Figure 4A). Like mutations at diagnosis, those at transformation were distributed throughout the protein, with a predilection for the FLD

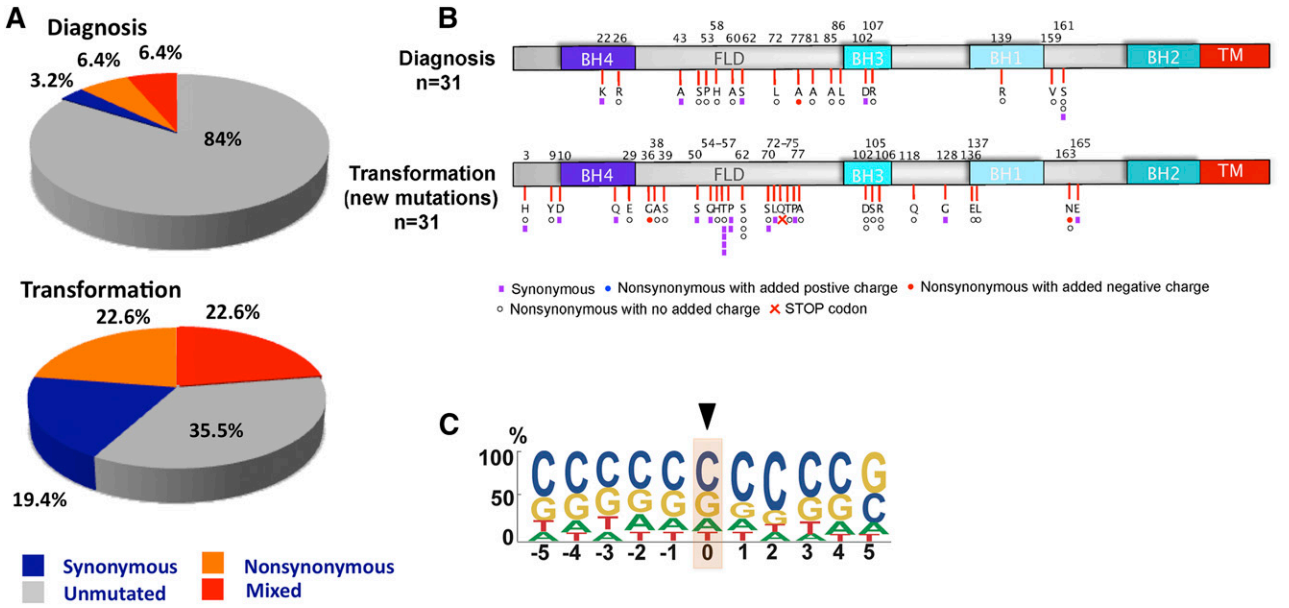


Figure 4. *BCL2* mutations at transformation in FL. (A) Overall frequency of *BCL2* somatic mutations in FL validation cohort at diagnosis and transformation. Gray, blue, orange, and red depict samples without mutations, with only synonymous mutations, with mixed synonymous and nonsynomymous mutations, and with only nonsynonymous mutations, respectively. (B) Schematic diagram of the Bcl-2 protein showing Bcl-2 homology domains and distribution of new amino acid alterations for FL validation samples (n = 31) at transformation. Color-coded symbols depict distinct types of alterations, with purple for synonymous, white for nonsynonymous with no charge introduction, red for nonsynonymous with introduction of a negative charge, and blue for nonsynonymous with introduction of a positive charge. Red cross identifies a stop codon. (C) Depiction of bp preference of mutations in the FL validation at diagnosis and transformation (n = 31). The size of letters corresponds to the frequency with which that nucleotide is mutated in the *BCL2* coding strand. bp, base pair; TM, transmembrane domain.

(Figure 4B) and a strong preference for Gs and Cs (79%) (Figure 4C). Nonetheless, the mutation signature at transformation was distinct from that at diagnosis (compare Figure 3A with Figure 4C), perhaps because 26 of the 31 patients had been treated with radiation or chemotherapy between diagnosis and transformation. Of the 5 FL patients not treated with a DNA damaging therapy prior to transformation, only 2 had *BCL2* mutations, a number that is too small to allow derivation of a therapy independent mutation signature at transformation.

Some FL *BCL2* mutations exhibit gain-of-function

Based on the nonrandom distribution and nonsynonymous nature of FL *BCL2* mutations, we began examining the effects of these mutations on Bcl-2 protein function. As illustrated in Figure 5 for the Bcl-2 G33R and A43T variants, products of some mutant *BCL2* alleles displayed increased affinity for immobilized Bim and Puma BH3 peptides in vitro (Figure 5A-B), as well as increased binding to these proapoptotic proteins in a cellular context (Figure 5C), consistent with *BCL2* gain-of-function mutations reported in some lymphoid cell lines.[46,47] Furthermore, in cell-based assays, these Bcl-2 variants offered more protection from apoptosis induced by Bim_{EL} overexpression (Figure 5D). The increased affinity for proapoptotic BH3 domains and enhanced protection was not, however, seen with all variant Bcl-2 proteins, as exemplified by Bcl-2 A82G.

BCL2 mutations correlate with FL clinical course

Examination of patient outcome in the FL discovery cohort revealed a striking decrease in overall survival from diagnosis if a *BCL2* mutation was present (Figure 6A). Further analysis examined the relationship between *BCL2* mutations and outcome in the validation cohort. As shown in Figure 6B, the presence of a *BCL2* mutation at diagnosis was associated with a marked increase in transformation risk compared with the absence of a *BCL2* mutation ($P < .0001$ by log-rank test; hazard ratio [HR] 3.56; 95% CI, 1.98-6.17). This association was even stronger than the relationship between AID expression and transformation risk (HR 1.70; 95% CI, 1.06-3.00; Figure 6C). These effects were most prominent in FLs with nonsynonymous mutations (Figure 6D). There was also a striking acceleration of death due to lymphoma (Figure 6E) in cases with *BCL2* mutations at diagnosis vs those without (median, 9.6 vs 20.4 years; $P = .012$ by log-rank test). In multivariate analysis, FLIPI and presence of *BCL2* mutations were independent risk factors for transformation, with HRs of 3.43 (95% CI, 1.67-6.66; $P = .0013$) and 3.61 (95% CI, 2.02-6.24; $P < .0001$), respectively. FLIPI and the presence of *BCL2* mutations were also independent risk factors for dying from lymphoma in multivariate analysis, with HRs of 2.67 (95% CI, 1.17-5.57; $P = .021$) and 2.28 (95% CI, 1.14-4.33; $P = .021$), respectively.

Discussion

In the present study, we report for the first time an association between *BCL2* mutations at diagnosis in FL, high AID expression, early disease progression, and poorer survival. These observations are potentially important for understanding FL transformation to clinically aggressive disease and the development of future therapeutic strategies.

Even though *BCL2* mutations have been previously reported in FL,[16,40,45,54,55] their correlation with clinical outcome has not been systematically explored. Several observations in the present study suggest that *BCL2* mutations might be functionally important. First, in our study, mutations were detected only in exon 2 and not in exons 1 or 3 (Figures 1B and 2A-C) even though the former would be closer to the *IGH* locus and more prone to AID-induced mutagenesis. Second, in marked contrast to DLBCL,[43] there was an excess of

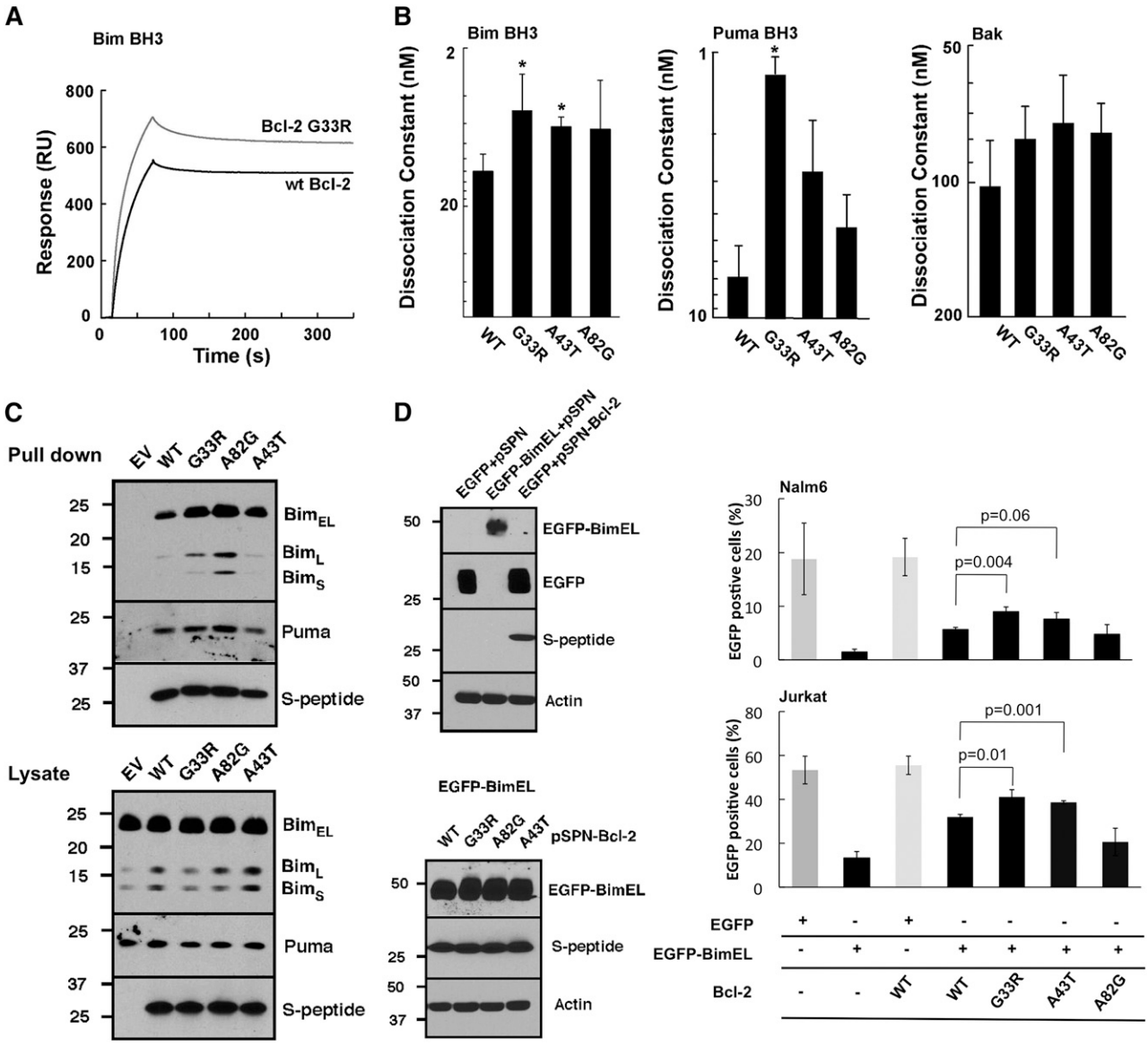


Figure 5. Some Bcl-2 amino acid substitutions increase affinity for proapoptotic family members. (A) Binding between immobilized human Bim BH3 peptide and 300 nM WT Bcl-2 or the G33R variant was analyzed by surface plasmon resonance. (B) Summary of dissociation constants for complexes of WT or mutant Bcl-2 with Bim BH3 peptide, Puma BH3 peptide, or BakΔTM protein showing that Bim and Puma BH3 domains are bound more tightly to Bcl-2 G33R or A43T than to WT. Error bars, SD of 3 independent experiments using different chips and protein preparations. *$P < .03$ vs WT sequence. (C) After cDNA encoding WT or variant Bcl-2 fused to S-peptide was expressed for 24 hours in Jurkat cells, S-peptide pulldowns (top) or whole cell lysates (bottom) were probed for S-peptide or the indicated Bcl-2 binding partners. (D) Nalm6 or Jurkat cells were transfected with plasmid encoding enhanced green fluorescent protein (EGFP) or EGFP fused to Bim$_{EL}$,[59] along with empty vector or plasmid encoding the indicated Bcl-2 variant. Cells were cultured for 24 hours in the presence of the broad spectrum caspase inhibitor Q-VD-OPh,[60] and subjected to immunoblotting to assess expression (left panels). Alternatively, after culture for 24 hours in the absence of Q-VD-OPh for 24 hours, cells were stained with APC-conjugated Annexin V and subjected to 2-color flow cytometry. Right panels show summary of EGFP-positive/Annexin V-negative cells (as a percentage of total cells) after transfection of Nalm6 cells (upper panel) or Jurkat cells (lower panel). Error bars, ± SD of 3 independent experiments. Procedures used for these in vitro studies were previously published[46,47,53,61] and are described in the supplemental Methods.

nonsynonymous mutations in FL (Figure 1E). Indeed, the vast majority of synonymous *BCL2* mutations in FL occur in association with nonsynonymous alterations (supplemental Table 3). Third, some of the mutant Bcl-2 proteins (eg, Bcl-2 G33R and Bcl-2 A43T), bound and neutralized proapoptotic Bcl-2 family members more effectively (Figure 5).

Although we have observed functional consequences of the *BCL2* mutations found in certain lymphoma cell lines[46,47] and in FL (eg, this study), it is important to emphasize that not all of the *BCL2* mutations are functionally significant. In particular, we have observed that the conservative A82G mutation has little impact on Bcl-2 function (Figure 5). Thus, the situation with *BCL2* mutations might be somewhat analogous to *BRCA1* or *BRCA2* missense mutations, where some are functionally important and others are not.[56]

Several additional observations suggest that AID might be involved in the generation of the *BCL2* SNVs. First, the vast majority of *BCL2* mutations occur at Cs and Gs, consistent with AID-induced cytidine deamination. Second, *BCL2* mutations exhibit a preference for AID target sequences rather than CpG sequences that would be preferred if spontaneous chemical deamination were responsible. Third, higher AID expression was observed in samples with *BCL2* mutations at diagnosis (Figure 3E).

When the relationship between AID, *BCL2* mutations, and survival was examined, transformation correlated with high AID

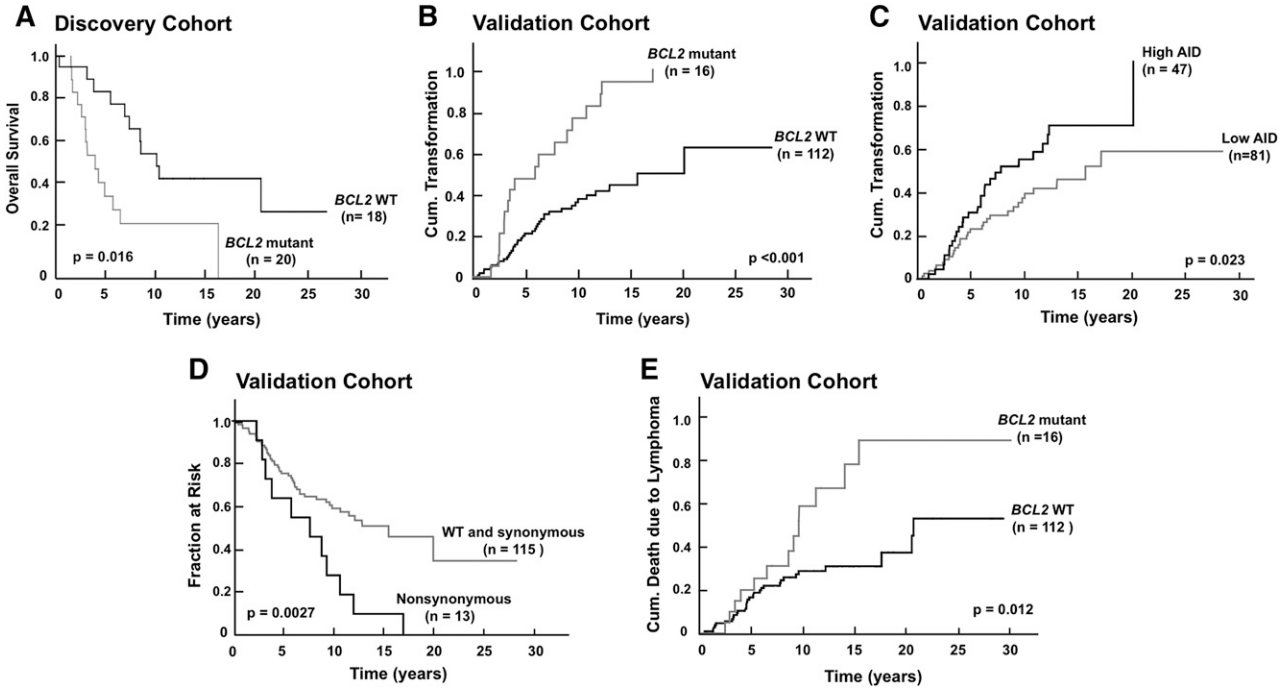


Figure 6. *BCL2* mutations correlate with transformation risk and disease-specific death in FL. (A) Kaplan-Meier plots showing impact of *BCL2* mutations on overall survival in FL discovery cohort. (B-C) Kaplan-Meier plots showing impact of *BCL2* mutations (B) or AID expression (C) on transformation risk in FL validation cohort. (D) Kaplan-Meier plots showing impact of *BCL2* nonsynonymous mutations on TTT in FL validation cohort. (E) Kaplan-Meier plots showing impact of *BCL2* mutations on death due to lymphoma in FL validation cohort.

expression (Figure 6C) and even more strongly with the presence of *BCL2* mutations. Initially observed in a prevalence study (Figure 6A), this relationship between *BCL2* mutations and earlier transformation was independently replicated in a larger independent study of FL samples, uniformly collected at diagnosis (Figure 6B). In short, the presence of *BCL2* mutations at diagnosis correlated with decreased TTT of FL and increased risk of death due to lymphoma (Figure 6B,E).

We also observed more *BCL2* mutations at transformation than at diagnosis (Figure 4), possibly reflecting ongoing mutagenic processes in FL. Comparison of the mutations at diagnosis and transformation nonetheless revealed potentially important differences. In particular, those acquired at transformation had a lower frequency of obvious AID signatures (15% at transformation vs 40% at diagnosis (supplemental Table 4), perhaps reflecting additional effects of intervening therapy or increased AID action at noncanonical sequences.[57] Moreover, some mutations at transformation were clearly nonfunctional (eg, a *BCL2* variant with a premature stop codon at transformation), again emphasizing that not all *BCL2* mutations enhance Bcl-2 protein function.

Despite the strong correlation between *BCL2* mutations at diagnosis and the poor outcome in 2 separate cohorts, we are aware of several limitations to the present study. First, the 2 cohorts differed from each other in important ways. The discovery cohort consisted of FLs with multiple cryopreserved vials decades after diagnosis. Consistent with the possibility that this cohort might have had bulkier disease, twice as many patients in the discovery cohort had FLIPI scores of 3 or 4 at diagnosis (Table 1). The frequency of mutations at diagnosis in this cohort also appeared higher, although the sample size was small.

Second, in order to have sufficient follow-up, the present study used cohorts treated almost exclusively in the prerituximab era. Accordingly, it will be important in the future to assess whether a similar correlation between *BCL2* mutation and poor outcome exists in patients receiving chemoimmunotherapy.

Third, because of our focus on sequence-associated changes in function of the Bcl-2 protein,[46,47] the present study differs from other recent efforts to catalog FL mutations. Sanger sequencing, a gold standard for detecting sequence variation, was used here. Moreover, the present study focused on *BCL2* exons, especially protein encoding exons 2 and 3. This more focused approach likely underestimates the total number of *BCL2* mutations compared with studies that used much more sensitive sequencing techniques and/or examined more noncoding *BCL2* sequence in an effort to identify all mutations in the *BCL2* locus.[16,44,45]

Finally, the present study does not address the question of whether *BCL2* mutations cause FL transformation. In our cohort, over 50% of transformed FLs have synonymous mutations or no mutations at all (Figure 4A), indicating that *BCL2* mutations are not required for FL transformation but leaving open the question of their contribution in transformed FLs that harbor them. In particular, we cannot rule out the possibility that *BCL2* mutations are simply a surrogate for AID-induced mutagenesis. AID has previously been implicated in mutating *BCL2*,[43] as well as *BCL6*, *MYC, PIM1*, and *PAX5*.[35,36] In the present study, *PIM1* mutations were less frequent than *BCL2* mutations and did not correlate with early transformation. Moreover, transformation correlated more strongly with *BCL2* mutation status (Figure 6B) than with AID expression (Figure 6C). Nonetheless, further evaluation of AID expression, *BCL2* mutation status, and disease progression in additional patient cohorts is required to further address this issue. Moreover, even if *BCL2* mutations are simply a reflection of AID activity, they might represent an objective and readily quantifiable measure of AID mutagenesis.

The observation that *BCL2* mutation status at diagnosis is an independent prognostic factor in FL that supplements the FLIPI has potentially important implications. A high FLIPI score has been

associated with transformation in multiple studies.[2,17,20,22,23,58] If poor outcome in patients with *BCL2*-mutant FL is also widely replicated, this might serve as the foundation for further studies in FL. In particular, the assessment of *BCL2* mutation status along with FLIPI could allow better stratification of FL patients in future clinical trials. In addition, it might also be reasonable to assess the effects of earlier therapeutic intervention, use of agents that bypass the mitochondrial apoptotic pathway, or treatment with Bcl-2 antagonists in this subgroup of FL patients.

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Authorship

Contribution: S.H.K., H. Dai, G.S.N., M.J.M., and J.R.C. conceptualized and designed the study; A.J.N., J.R.C., S.L.S., T.M.H., J.E.K., S.D.G., N.E.K., and T.E.W. provided study material or patients; C.C., P.A.S., H. Dai, A.L.F., H. Ding, X.W.M., A.D., A.K.C., X.W., W.R.M., and T.M.H. collected and assembled the data; C.C., M.J.M., D.F.J., G.S.N., and S.H.K. analyzed and interpreted the data; S.H.K., G.S.N., and T.E.W. provided financial support for the study; and all authors wrote and gave final approval of the manuscript.

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References

1. Lunning MA, Vose JM. Management of indolent lymphoma: where are we now and where are we going. *Blood Rev.* 2012;26(6):279-288.
2. Sehn LH, Fenske TS, Laport GG. Follicular lymphoma: prognostic factors, conventional therapies, and hematopoietic cell transplantation. *Biol Blood Marrow Transplant.* 2012;18(suppl 1): S82-S91.
3. Ujjani C, Cheson BD. The optimal management of follicular lymphoma: an evolving field. *Drugs.* 2013;73(13):1395-1403.
4. Hubbard SM, Chabner BA, DeVita VT Jr, et al. Histologic progression in non-Hodgkin's lymphoma. *Blood.* 1982;59(2):258-264.
5. Acker B, Hoppe RT, Colby TV, Cox RS, Kaplan HS, Rosenberg SA. Histologic conversion in the non-Hodgkin's lymphomas. *J Clin Oncol.* 1983; 1(1):11-16.
6. Garvin AJ, Simon RM, Osborne CK, Merrill J, Young RC, Berard CW. An autopsy study of histologic progression in non-Hodgkin's lymphomas. 192 cases from the National Cancer Institute. *Cancer.* 1983;52(3):393-398.
7. Horning SJ, Rosenberg SA. The natural history of initially untreated low-grade non-Hodgkin's lymphomas. *N Engl J Med.* 1984;311(23): 1471-1475.
8. Bastion Y, Sebban C, Berger F, et al. Incidence, predictive factors, and outcome of lymphoma transformation in follicular lymphoma patients. *J Clin Oncol.* 1997;15(4):1587-1594.
9. Giné E, Montoto S, Bosch F, et al. The Follicular Lymphoma International Prognostic Index (FLIPI) and the histological subtype are the most important factors to predict histological transformation in follicular lymphoma. *Ann Oncol.* 2006;17(10):1539-1545.
10. Montoto S, Davies AJ, Matthews J, et al. Risk and clinical implications of transformation of follicular lymphoma to diffuse large B-cell lymphoma. *J Clin Oncol.* 2007;25(17):2426-2433.
11. Al-Tourah AJ, Gill KK, Chhanabhai M, et al. Population-based analysis of incidence and outcome of transformed non-Hodgkin's lymphoma. *J Clin Oncol.* 2008;26(32):5165-5169.
12. Nagy M, Balázs M, Adám Z, et al. Genetic instability is associated with histological transformation of follicle center lymphoma. *Leukemia.* 2000;14(12):2142-2148.
13. Li L, Gupta S, Bashir T, Koduru PR, Brody J, Allen SL. Serial cytogenetic alterations resulting in transformation of a low-grade follicular lymphoma to Burkitt lymphoma. *Cancer Genet Cytogenet.* 2006;170(2): 140-146.
14. Maeshima AM, Omatsu M, Nomoto J, et al. Diffuse large B-cell lymphoma after transformation from low-grade follicular lymphoma: morphological, immunohistochemical, and FISH analyses. *Cancer Sci.* 2008;99(9): 1760-1768.
15. Schatz JH, Oricchio E, Puvvada SD, Wendel HG. Progress against follicular lymphoma. *Curr Opin Hematol.* 2013;20(4):320-326.
16. Pasqualucci L, Khiabanian H, Fangazio M, et al. Genetics of follicular lymphoma transformation. *Cell Reports.* 2014;6(1):130-140.
17. Wong E, Dickinson M. Transformation in follicular lymphoma: biology, prognosis, and therapeutic options. *Curr Oncol Rep.* 2012;14(5):424-432.
18. Bernstein SH, Burack WR. The incidence, natural history, biology, and treatment of transformed lymphomas. *Hematology (Am Soc Hematol Educ Program).* 2009;532-541.
19. Montoto S, Fitzgibbon J. Transformation of indolent B-cell lymphomas. *J Clin Oncol.* 2011; 29(14):1827-1834.
20. Link BK, Maurer MJ, Nowakowski GS, et al. Rates and outcomes of follicular lymphoma transformation in the immunochemotherapy era: a report from the University of Iowa/MayoClinic Specialized Program of Research Excellence Molecular Epidemiology Resource. *J Clin Oncol.* 2013;31(26):3272-3278.
21. Solal-Céligny P, Roy P, Colombat P, et al. Follicular lymphoma international prognostic index. *Blood.* 2004;104(5):1258-1265.
22. Relander T, Johnson NA, Farinha P, Connors JM, Sehn LH, Gascoyne RD. Prognostic factors in follicular lymphoma. *J Clin Oncol.* 2010;28(17): 2902-2913.
23. Bello C, Zhang L, Naghashpour M. Follicular lymphoma: current management and future directions. *Cancer Contr.* 2012;19(3):187-195.
24. Nogai H, Dörken B, Lenz G. Pathogenesis of non-Hodgkin's lymphoma. *J Clin Oncol.* 2011;29(14): 1803-1811.
25. Kridel R, Sehn LH, Gascoyne RD. Pathogenesis of follicular lymphoma. *J Clin Invest.* 2012; 122(10):3424-3431.
26. Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature.* 1988;335(6189):440-442.
27. Strasser A, Cory S, Adams JM. Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. *EMBO J.* 2011;30(18):3667-3683.
28. Brunelle JK, Letai A. Control of mitochondrial apoptosis by the Bcl-2 family. *J Cell Sci.* 2009; 122(pt 4):437-441.
29. Llambi F, Moldoveanu T, Tait SW, et al. A unified model of mammalian BCL-2 protein family interactions at the mitochondria. *Mol Cell.* 2011; 44(4):517-531.
30. Yang E, Korsmeyer SJ. Molecular thanatopsis: a discourse on the BCL2 family and cell death. *Blood.* 1996;88(2):386-401.
31. Stavnezer J, Guikema JE, Schrader CE. Mechanism and regulation of class switch recombination. *Annu Rev Immunol.* 2008;26: 261-292.
32. Lossos IS, Levy R, Alizadeh AA. AID is expressed in germinal center B-cell-like and activated B-cell-like diffuse large-cell lymphomas and is not correlated with intraclonal heterogeneity. *Leukemia.* 2004;18(11):1775-1779.
33. Di Noia JM, Neuberger MS. Molecular mechanisms of antibody somatic hypermutation. *Annu Rev Biochem.* 2007;76:1-22.
34. Pasqualucci L, Bhagat G, Jankovic M, et al. AID is required for germinal center-derived lymphomagenesis. *Nat Genet.* 2008;40(1): 108-112.
35. Halldórsdóttir AM, Frühwirth M, Deutsch A, et al. Quantifying the role of aberrant somatic hypermutation in transformation of follicular lymphoma. *Leuk Res.* 2008;32(7):1015-1021.
36. Bödör C, Bognár A, Reiniger L, et al. Aberrant somatic hypermutation and expression of activation-induced cytidine deaminase mRNA in

mediastinal large B-cell lymphoma. *Br J Haematol.* 2005;129(3):373-376.

37. Pérez-Durán P, de Yebenes VG, Ramiro AR. Oncogenic events triggered by AID, the adverse effect of antibody diversification. *Carcinogenesis.* 2007;28(12):2427-2433.
38. Robbiani DF, Bothmer A, Callen E, et al. AID is required for the chromosomal breaks in c-myc that lead to c-myc/IgH translocations. *Cell.* 2008; 135(6):1028-1038.
39. Seto M, Jaeger U, Hockett RD, et al. Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. *EMBO J.* 1988;7(1):123-131.
40. Tanaka S, Louie DC, Kant JA, Reed JC. Frequent incidence of somatic mutations in translocated BCL2 oncogenes of non-Hodgkin's lymphomas. *Blood.* 1992;79(1):229-237.
41. Reed JC, Tanaka S. Somatic point mutations in the translocated bcl-2 genes of non-Hodgkin's lymphomas and lymphocytic leukemias: implications for mechanisms of tumor progression. *Leuk Lymphoma.* 1993;10(3): 157-163.
42. McDonnell TJ, Korsmeyer SJ. Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). *Nature.* 1991;349(6306):254-256.
43. Lohr JG, Stojanov P, Lawrence MS, et al. Discovery and prioritization of somatic mutations in diffuse large B-cell lymphoma (DLBCL) by whole-exome sequencing. *Proc Natl Acad Sci USA.* 2012;109(10):3879-3884.
44. Schuetz JM, Johnson NA, Morin RD, et al. BCL2 mutations in diffuse large B-cell lymphoma. *Leukemia.* 2012;26(6):1383-1390.
45. Okosun J, Bödör C, Wang J, et al. Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. *Nat Genet.* 2014;46(2):176-181.
46. Dai H, Meng XW, Lee S-H, Schneider PA, Kaufmann SH. Context-dependent Bcl-2/Bak interactions regulate lymphoid cell apoptosis. *J Biol Chem.* 2009;284(27):18311-18322.
47. Smith AJ, Dai H, Correia C, et al. Noxa/Bcl-2 protein interactions contribute to bortezomib resistance in human lymphoid cells. *J Biol Chem.* 2011;286(20):17682-17692.
48. Kaplan E, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc.* 1958;53:457-481.
49. Cheson BD, Pfistner B, Juweid ME, et al; International Harmonization Project on Lymphoma. Revised response criteria for malignant lymphoma. *J Clin Oncol.* 2007;25(5): 579-586.
50. Weber JS, Berry J, Litwin S, Claflin JL. Somatic hypermutation of the JC intron is markedly reduced in unrearranged kappa and H alleles and is unevenly distributed in rearranged alleles. *J Immunol.* 1991;146(9):3218-3226.
51. Deng X, Gao F, Flagg T, May WS Jr. Mono- and multisite phosphorylation enhances Bcl2's antiapoptotic function and inhibition of cell cycle entry functions. *Proc Natl Acad Sci USA.* 2004; 101(1):153-158.
52. Jin Z, May WS, Gao F, Flagg T, Deng X. Bcl2 suppresses DNA repair by enhancing c-Myc transcriptional activity. *J Biol Chem.* 2006; 281(20):14446-14456.
53. Dai H, Ding H, Meng XW, Lee S-H, Schneider PA, Kaufmann SH. Contribution of Bcl-2 phosphorylation to Bak binding and drug resistance. *Cancer Res.* 2013;73(23):6998-7008.
54. Matolcsy A, Casali P, Warnke RA, Knowles DM. Morphologic transformation of follicular lymphoma is associated with somatic mutation of the translocated Bcl-2 gene. *Blood.* 1996;88(10): 3937-3944.
55. Green MR, Gentles AJ, Nair RV, et al. Hierarchy in somatic mutations arising during genomic evolution and progression of follicular lymphoma. *Blood.* 2013;121(9):1604-1611.
56. Guidugli L, Carreira A, Caputo SM, et al; ENIGMA consortium. Functional assays for analysis of variants of uncertain significance in BRCA2. *Hum Mutat.* 2014;35(2):151-164.
57. Duquette ML, Huber MD, Maizels N. G-rich proto-oncogenes are targeted for genomic instability in B-cell lymphomas. *Cancer Res.* 2007;67(6): 2586-2594.
58. Freedman A. Follicular lymphoma: 2012 update on diagnosis and management. *Am J Hematol.* 2012;87(10):988-995.
59. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell.* 1999; 3(3):287-296.
60. Caserta TM, Smith AN, Gultice AD, Reedy MA, Brown TL. Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. *Apoptosis.* 2003;8(4):345-352.
61. Dai H, Pang Y-P, Ramirez-Alvarado M, Kaufmann SH. Evaluation of the BH3-only protein Puma as a direct Bak activator. *J Biol Chem.* 2014;289(1):89-99.