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Original contribution

Decreased BIM expression in BCL2-negative follicular lymphoma: a potential mechanism for resistance to apoptosis☆,☆☆,☆☆☆

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Summary Follicular lymphoma (FL) is characterized by t(14; 18)(q32; q21), leading to overexpression of the antiapoptotic molecule BCL2; however, a subset of FLs lack *BCL2* rearrangement and BCL2 expression as analyzed by immunohistochemistry (IHC). In this study, we evaluated expression of antiapoptotic (MCL1 and BCL-XL) and proapoptotic proteins (BIM) by IHC in both BCL2(−) and BCL2(+) FLs. FLs diagnosed between 2009 and 2019 were reviewed to identify BCL2(−) cases by IHC (assessed by clone 124). Immunohistochemical analyses for BCL2 (EP36), MCL1, BIM, BCL-XL, and Ki-67 were performed on tissue microarrays or whole slides. BCL2 (EP36) was interpreted as positive (≥10%) or negative (<10%). Ki-67 was interpreted on tumor cells in 10% increments. The remaining immunohistochemical analysis results were scored on tumor cells in 10% increments, and intensity was interpreted as weak, moderate, or strong to derive an H-score. Twenty-four BCL2(−) FLs were initially identified, but on further testing with BCL2(EP36) immunohistochemical staining, 5 of 24 were reclassified as BCL2(+), leaving 19 BCL2(−) FLs. Thirty-three BCL2(+) FLs were selected with sufficient tissue for additional immunohistochemical analyses. There was no significant difference in expression of antiapoptotic BCL-XL or MCL1 between BCL2(−) and BCL2(+) FLs ($p = 0.75$ and 0.28, respectively). However, proapoptotic BIM expression was significantly lower in BCL2(−) FLs than in BCL2(+) FLs ($p = 0.002$). In our study, 21% of putative BCL2(−) FLs were BCL2(+) when tested with alternative clones, supporting the practice of having more than one BCL2 clone in immunohistochemical laboratories. Decreased BIM expression in BCL2(−) FLs could have an

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overall antiapoptotic effect and represent an alternate mechanism for cell survival in BCL2(−) FLs.

1. Introduction

Follicular lymphoma (FL) is the most common indolent B-cell lymphoma in the United States and western Europe, representing approximately 20% of all lymphomas [1]. FLs usually demonstrate a germinal center B-cell immunophenotype (coexpression of CD20, CD10, and BCL6) with aberrant expression of BCL2 protein within neoplastic follicles. Overexpression of BCL2 protein is usually a consequence of t(14; 18)(q32; q21), juxtaposing *BCL2* and *IGH* [1]. This is a main molecular driver in the pathogenesis of FL, and expression of BCL2 in follicle center B cells is a feature that is routinely assessed in the diagnosis of FL. However, a subset of FLs, particularly high cytologic grade cases, lack *BCL2* rearrangement and BCL2 protein expression as assessed by immunohistochemistry (IHC) [2].

Alternate mechanisms by which BCL2(−) FLs might compensate for lack of BLC2 overexpression have not been well studied. Although there are many additional BCL2 family member proteins that have antiapoptotic effects (BCL2, BCL-XL, BCL-w, BCL2A1, and MCL1) and proapoptotic effects (BAX, BAK, and the BH3-only molecules BIM, BAD, BMF, NOXA, HRK, PUMA, BIK, and BID), we were interested particularly in BCL-XL, MCL1, and BIM. BCL-XL is expressed in many B-cell lymphomas including FL and has been relevant in FL models in which induction of apoptosis was associated with decreased BCL-XL expression [3,4]. Similarly, MCL1 has been studied in FL, and when expressed, it can block B-cell receptor−induced apoptosis in t(14; 18)-positive cell lines [5]. In addition, MCL1-transgenic mice develop FL-like lesions [6]. Furthermore, MCL1 expression has been associated with poor prognosis in FL [7]. With regard to BIM, we have previously shown levels of BIM are relevant to apoptosis resistance mechanisms in t(14; 18)-positive cell lines [8].

In this study, we evaluated a series of BCL2(−) FLs and compared them with a set of BCL2(+) FLs for BCL-XL, MCL1, and BIM expression as well as the proliferation marker Ki-67 to determine whether differences in expression of these markers might compensate for lack of BCL2. This might also have implications in alternate strategies for targeted therapies in such cases.

2. Materials and methods

2.1. Case selection

This study was approved by our institutional review board. Cases diagnosed as FL between 2009 and 2019 were retrieved from our anatomic pathology archives by computerized search. Pathology reports were reviewed to identify BCL2(−) FLs (by immunohistochemistry) and grade-matched BCL2(+) FLs to serve as a control group. Cases with *BCL2* rearrangement as analyzed by conventional cytogenetics or fluorescence in situ hybridization (FISH) (if performed) were excluded from the study group, but were allowed in the control group. Pre-existing hematoxylin and eosin−stained slides, immunohistochemical staining results, and clinical data were reviewed to confirm the initial diagnosis. Cases with insufficient tissue remaining to perform additional immunohistochemical analyses were excluded.

2.2. Immunohistochemistry

Tissue microarrays were constructed from paraffin tissue blocks when sufficient tissue was available for punching. In recently diagnosed cases and cases with limited tissue, whole-slide sections were used to preserve the tissue block. Immunohistochemical analyses for BCL2 (clone EP36), Ki-67, MCL1, BIM, and BCL-XL were performed at Cleveland Clinic on formalin-fixed paraffin-embedded tissue sections cut at 4-micron thickness. Details regarding antibody clone, dilution, and antigen retrieval process are outlined in Table 1. Expression of BCL2 within germinal centers on ≥10% of neoplastic B cells was considered positive. Ki-67 was scored within follicles in 10% increments (0−100%). The remaining analysis results were evaluated within follicles for both intensity (weak, moderate, or strong) and percentage of positive cells (10% increments). The combination of intensity and percentage was used to calculate an H-score as follows: 3 × percentage of strongly staining nuclei + 2 × percentage of moderately staining nuclei + percentage of weakly staining nuclei, giving a range of 0−300.

2.3. Fluorescence in situ hybridization

Data regarding FISH studies for rearrangement of *BCL2*, *BCL6*, *MYC*, and/or *IGH/MYC* fusion performed during routine workup of the selected FLs were collected. Cases with rearrangement of *BCL2* were excluded from the study group of BCL2(−) FLs, but were allowed in the control group of BCL2(+) FLs.

Interphase FISH analysis was performed at the time of diagnosis at Cleveland Clinic using the following probes (Abbott Molecular, Des Plaines, IL): LSI *MYC* dual-color, break-apart rearrangement probes; LSI *IGH/MYC/CEP8* tricolor, dual-fusion probes; LSI *BCL2* dual-color, break-apart rearrangement probes; LSI *IGH/BCL2* dual-color,

Table 1 IHC technical data.

Immunohistochemistry technical information				
Target	Ab source	Ab clone	Dilution	Antigen retrieval
BCL2	Cell Marque	EP36	1:100	Cell conditioning solution 1 (10 min, 37°C)
Ki-67	Ventana Medical Systems	30−9	Prediluted	Cell conditioning solution 1 (32 min, 37°C)
MCL1	Cell Signaling Technology	D5V5L	1:50	Cell conditioning solution 1 (40 min, 90°C)
BIM	Cell Signaling Technology	C34C5	1:50	Cell conditioning solution 1 (60 min, 95°C)
BCL-XL	Cell Signaling Technology	54H6	1:2000	Cell conditioning solution 1 (24 min, 95°C)

NOTE. A summary of technical information for immunohistochemical stains used in this study. All staining procedures were performed on the Ventana Benchmark Ultra (Ventana Medical Systems, Tucson, AZ).
Abbreviations: IHC, immunohistochemistry.

dual-fusion translocation probes; and LSI *BCL6* dual-color, break-apart rearrangement probes. Reference ranges varied depending on the FISH probe.

2.4. Conventional cytogenetics

Data regarding conventional cytogenetic studies performed during the routine workup of the selected FLs were collected. Cases with cytogenetic evidence of t(14; 18)(q32; q21) or variant translocations involving the *BCL2* locus were excluded from the study group of BCL2(−) FLs, but were allowed in the control group of BCL2(+) FLs.

2.5. Clonality (BIOMED-2)

Data regarding clonality studies performed during the routine workup of the selected FLs were collected. In brief, DNA was isolated from the specimen and subjected to polymerase chain reaction (PCR) amplification using a fluorescently labeled primer targeting the immunoglobulin heavy chain (IGH) and immunoglobulin light chain kappa (IGK) loci (Invivoscribe, San Diego, CA). Fluorescently labeled PCR products were analyzed by capillary gel electrophoresis. An additional PCR reaction directed at housekeeping genes was performed as a control for each sample to ensure adequate DNA quality.

2.6. BIM image analysis

Sections labeled with BIM antibody were scanned using the Aperio slide scanner (Leica Biosystems, Buffalo Grove, IL). H-scores were acquired using a modified Cytoplasmic V2 algorithm in ImageScope software (Leica Biosystems).

2.7. Statistical analysis

Statistical analyses (Fisher's exact test, Mann-Whitney U test, and Spearman rank-order correlation) were performed using Statistica (StatSoft, Inc., Tulsa, OK), with a p-value of <0.05 considered significant.

3. Results

3.1. Patient demographics

Twenty-four BCL2(−) FL specimens were identified with sufficient tissue for additional immunohistochemical analyses. Upon repeat testing with BCL2(EP36) IHC, 5 of 24 BCL2(−) FLs were reclassified as BCL2(+) FLs, leaving 19 specimens from 19 patients that were negative with both BCL2 antibody clones. Of the 19 BCL2(−) FL specimens, sites included the lymph node (N = 13, 68.4%), extranodal sites (N = 5, 26.3%), and the spleen (N = 1, 5.3%). Nodal locations included the following: inguinal/groin (N = 5), cervical (N = 2), axillary (N = 1), submandibular (N = 1), supraclavicular (N = 1), submental (N = 1), mesenteric (N = 1), and periparotid (N = 1). Extranodal sites included the following: mesenteric mass (N = 1), acromion (N = 1), thyroid (N = 1), breast (N = 1), and orbit (N = 1).

Thirty-three BCL2(+) FL specimens from 32 patients (2 specimens were from the same patient at different locations and time points) were selected with sufficient tissue for additional immunohistochemical analyses. Of the 33 BCL2(+) FL specimens, sites included nodal sites (N = 20, 60.6%), extranodal sites (N = 12, 36.4%), and the spleen (N = 1, 3.0%). Nodal locations included the following: cervical (N = 7), inguinal (N = 5), axillary (N = 4), submandibular (N = 2), mesenteric (N = 1), and retroperitoneal (N = 1). Extranodal sites included the following: tonsil/base of the tongue (N = 2), parotid (N = 2), bladder (N = 1), gastrointestinal polyp (N = 1), mediastinum (N = 1), retroperitoneal mass (N = 1), pelvic mass (N = 1), conjunctiva (N = 1), chest wall (N = 1), and mesenteric mass (N = 1). There was no significant difference in presenting location (nodal versus extranodal) between BCL2(−) and BCL2(+) FLs (p = 0.898, Fisher's exact test).

There was no significant difference in patient demographics, with a mean age of 64.7 and 63.9 years and a male:female ratio of 0.7 and 1.1 for BCL2(−) and BCL2(+) FLs, respectively (p = 0.761 and 0.517, respectively, Mann-Whitney U test and Fisher's exact test).

The majority of cases in both BCL2(−) and BCL2(+) groups were of grade 1−2, with fewer grade 3 cases. The BCL2(−) FL group showed a similar distribution of grade 1−2, grade 3A, and grade 3B cases as the BCL2(+) group (Table 2).

In the BCL2(−) FL group, 3 of 19 patients had a prior history of lymphoma including FL (N = 2) and unspecified B-cell non-Hodgkin lymphoma (NHL) (N = 1). Both FLs were negative for BCL2 at initial diagnosis. The unspecified B-cell NHL was diagnosed at another institute, and slides were not available for review. Prior treatment for these 3 patients included R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone) (N = 2) and single-agent rituximab (N = 1). After recurrence, two of these patients are being observed, and one has received two additional lines of therapy. Sixteen of 19 patients in the BCL2(−) FL group were newly diagnosed, and treatment included active surveillance (N = 4), single-agent rituximab (N = 3), unknown/lost to follow-up (N = 3), radiotherapy only (N = 2), chemotherapy including R-CHOP or BR (bendamustine and rituximab) (N = 2), ≥3 lines of therapy (N = 1), and combined

Table 2 Patient demographic and ancillary testing data.

Demographic and ancillary testing data			
	BCL2(−) FL (N = 19)	BCL2(+) FL (N = 33)	P-value
Mean age (years)	64.7	63.9	NS[a]
Male:female ratio	0.7	1.1	NS
Grade			
1/2	12 (63.1%)	21 (63.6%)	NS
3A	6 (31.6%)	10 (30.3%)	NS
3B	1 (5.3%)	2 (6.1%)	NS
Prior lymphoma	3 (15.8%)	9 (27.2%)	NS
FL	2	4	
DLBCL	0	3	
FL + DLBCL	0	2	
B-cell NHL (unspecified)	1	0	
Treatment			
Active surveillance	4	10	
Single-agent rituximab	4	1	
Chemotherapy	3	9	
Radiation only	2	1	
Chemotherapy + radiation	1	2	
Multiple lines (≥3)	2	7	
Unknown/lost to follow-up	3	2	
Other	0	1	
Survival			
Alive	15 (78.9%)	27 (84.4%)	NS
Dead	2 (10.5%)	2 (6.3%)	
Lost to follow-up	2 (10.5%)	3 (9.4%)	
Clonality (BIOMED-2)	6 (31.6%)	0 (0%)	
Positive	5	N/A	
Negative	1	N/A	
FISH studies	7 (36.8%)	0 (0%)	
BCL2 break-apart	2	N/A	
BCL2-IgH fusion	2	N/A	
BCL2, BCL6, MYC, and *MYC/IGH*	2	N/A	
BCL2 and *BCL6* break-apart	1	N/A	
Cytogenetics	3 (15.7%)	8 (24.2%)	NS
Normal	1	0	
t(14;18)	0	6	
Variant *BCL2* translocation	0	1	
Abnormal (other)	1	1	
No growth	1	0	

NOTE. A comparison of demographic information, ancillary testing data, and treatment/survival data between BCL2(−) and BCL2(+) FLs. There is no significant difference in mean age, male:female ratio, grade, percentage of patients with prior lymphoma, or overall survival.

Abbreviations: FL, follicular lymphoma; FISH, fluorescence in situ hybridization; NHL, non-Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma.

[a] NS = not statistically significant (P > 0.05).

rituximab and radiotherapy (N = 1). In the BCL2(−) FL group, after a mean follow-up time of 31.4 months, 15 patients were alive (overall survival = 78.9%), two were lost to follow-up, and two were dead (both unrelated to lymphoma).

In the BCL2(+) FL group, 8 of 32 patients had a prior history of lymphoma including diffuse large B-cell lymphoma (DLBCL) (N = 3), FL (N = 3), and DLBCL arising in FL (N = 2). Prior treatment for these 8 patients included R-CHOP or BR (N = 5), radiotherapy alone (N = 2), and active surveillance (N = 1). After recurrence, five of these patients went on to receive at least three lines of therapy, two were treated with chemotherapy alone (BR or R-CHOP), and one was observed. Twenty-four of 32 patients in the BCL2(+) FL group were newly diagnosed, and treatment included active surveillance (N = 10), chemotherapy including R-CHOP or BR (N = 7), ≥3 lines of therapy (N = 2), unknown/lost to follow-up (N = 2), radiotherapy only (N = 1), single-agent rituximab (N = 1), and no therapy secondary to death (N = 1). In the BCL2(+) FL group, after a mean follow-up time of 13.9 months, 27 patients were alive (overall survival = 84.4%), 3 were lost to follow-up, and 2 were dead (1 from progressive lymphoma and 1 unrelated to lymphoma). Further analysis of survival data was not performed owing to short follow-up time and a history of lymphoma and/or treatment in a subset of patients. Additional demographic and ancillary testing data are presented in Table 2.

3.2. Immunohistochemical results

Of the originally selected BCL2(−) FLs (N = 24), 5 demonstrated expression of BCL2 with an alternate antibody clone (EP-36), leaving 19 FL specimens negative with both BCL2 antibody clones. Comparing these 19 BCL2(−) FLs with the BCL2(+) control group, there were no differences in expression of BCL-XL or MCL1 expression. Similarly, there was no difference in Ki-67 index or proportion of cases with high (>30%) Ki67 index. However, BIM expression levels were significantly lower in BCL2(−) FLs (mean H-score for BIM = 50.6 ± 38.8) than in BCL2(+) FLs (87.7 ± 56.8), p = 0.002 (Mann-Whitney U test). Similarly, the percentage of cells positive for BIM (without regard to intensity) was significant lower in the BCL2(−) FL group (mean percentage of BIM expression = 26.1% ± 16.2%) than in BCL2(+) FLs (44.1% ± 20.5%), p = 0.004 (Mann-Whitney U test). These results are summarized in Table 3. Representative examples of immunohistochemical expression in BCL2(−) and BCL2(+) FLs are shown in Fig. 1.

To confirm the BIM result, image analysis was performed on all BIM-stained slides from both the BCL2(−) FL group and the BCL2(+) FL group. By image analysis, the BIM H-score was significantly lower in the BCL2(−) group than in the BCL2(+) group (mean H-score = 66.7 versus 129.1, respectively), p = 0.0015 (Mann-Whitney U test). There was significant correlation between digital and manual scoring (R = 0.86), p < 0.001 (Spearman rank-order test).

Table 3 Immunohistochemical expression data.

Mean scores ± 1 SD	BCL2(−) FL	BCL2(+) FL	P-value
BCL-XL (H-score)	60.9 ± 52.0	66.6 ± 40.8	0.753
MCL1 (H-score)	50.9 ± 53.0	34.1 ± 38.1	0.281
BIM (H-score)	50.6 ± 38.8	87.7 ± 56.8	0.002
BIM (%)	26.1 ± 16.2	44.1 ± 20.5	0.004
Ki-67 (%)	37.0 ± 27.7	43.2 ± 21.0	0.177[a]

NOTE. A comparison of the mean H-scores ± 1 SD (BCL-XL, MCL1, and BIM) and mean percentage ± 1 SD (Ki-67 and BIM) between BCL2(−) FLs and BCL2(+) FLs.

Abbreviations: FL, follicular lymphoma; SD, standard deviation.

[a] When considering Ki67 high (≥30% cutoff), there was no difference in the proportion of cases with a high proliferative index between BCL2(−) and BCL2(+) FLs (P = 0.45).

3.3. Molecular genetic and karyotypic features

Clonality studies (BIOMED-2 protocol) were performed in 6 of 19 (31.6%) BCL2(−) FLs, with 5 demonstrating clonal rearrangements and one showing no evidence of clonality. FISH studies were performed in 7 of 19 (36.8%) BCL2(−) FLs. FISH studies included the following: *BCL2* break-apart only (N = 2); *BCL2/IGH* fusion only (N = 2); a B-cell lymphoma FISH panel consisting of *BCL2* break-apart, *BCL6* break-apart, *MYC* break-apart, and *MYC/IGH* fusion (N = 2); and both *BCL2* and *BCL6* break-apart probes (N = 1). All FISH studies were negative for rearrangements at all tested loci. Conventional cytogenetics were performed in 3 of 19 BCL2(−) FLs (15.8%). One case demonstrated a normal male karyotype, one case had no growth, and one had an abnormal karyotype but no evidence of rearrangement involving the *BCL2* locus.

4. Discussion

FL is typified by BCL2 protein overexpression, usually secondary to t(14; 18)(q32; q21). This is considered the genetic hallmark of FL, and overexpression confers a survival advantage to FL cells owing to apoptosis resistance. Indeed, early transgenic mouse models harboring the *IGH-BCL2* rearrangement develop follicular hyperplasia, and a subset progresses to lymphoma [9]. Interestingly, a significant minority of FLs are negative for BCL2 protein and show no evidence of *BCL2* rearrangement [1,10]. Although a subset of these BCL2(−) FLs may harbor *BCL6* rearrangements, most (90%) do not, and the pathogenic mechanisms for apoptosis resistance and lymphomagenesis are poorly understood [11]. Prior studies have highlighted differences at the molecular level in BCL2(−) FLs

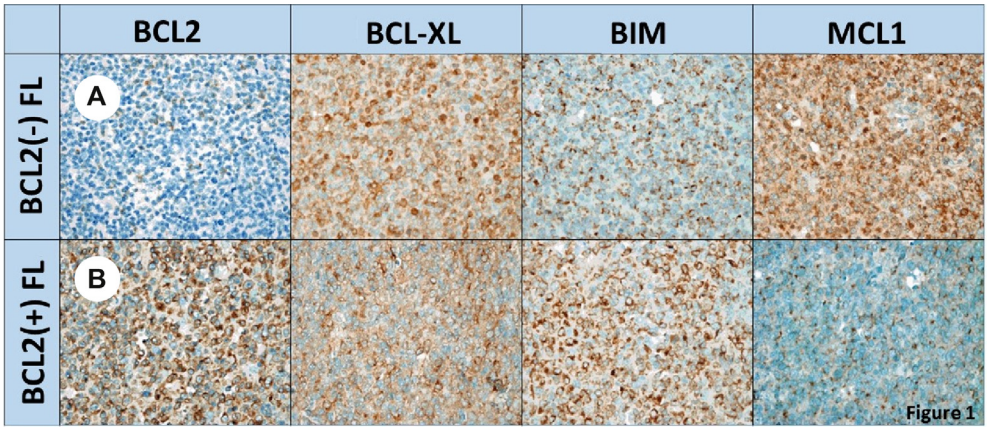

Fig. 1 BCL2 family immunohistochemistry in FL. BCL2(−) FL (A) and BCL2(+) FL (B). Immunohistochemical staining results for BCL2 (clone EP36), BCL-XL, BIM, and MCL1 (all images at ×400). There is similar expression of BCL-XL between the BCL2(−) and BCL2(+) FLs. There is decreased expression of BIM in the BCL2(−) FL. There is increased expression of MCL1 in the BCL2(−) FL. FL, follicular lymphoma.

including gains of 2p16, containing the *BCL11A* and *REL* loci; enrichment of activated B-cell−like or late germinal center B-cell−like signatures; and decreased expression of miR-16, leading to overexpression of CHEK1 and an overall antiapoptotic state [12,13]. There are 18 BCL2 family proteins that are subdivided into antiapoptotic (BCL2, BCL-XL, BCL-w, BCL2A1, and MCL1), proapoptotic multidomain (BAX and BAK), and proapoptotic BH3-only proteins (BIM, BAD, BMF, NOXA, HRK, PUMA, BIK, and BID) [14]. We hypothesized that differential expression of one or more of these BCL2 family proteins could lead to a similar antiapoptotic state as seen in BCL2(+) FLs. We chose to investigate BIM, MCL1, and BCL-XL because of prior studies demonstrating their relevance to hematolymphoid neoplasms including FL [3−8].

Although a significant subset of FLs are negative for BCL2 protein as per IHC, some of these still harbor *BCL2* rearrangements, but have additional *BCL2* mutations that alter the immunohistochemical binding site epitope [15−17]. Correct identification of these cases can be achieved by using alternate antibody clones with specificity for different BCL2 epitopes or by using *BCL2* FISH studies [18]. In our study, 21% of putative BCL2(−) FLs expressed BCL2 when reassessed with an alternate antibody clone (EP36), demonstrating the importance of having alternate BCL2 antibodies routinely available in immunohistochemical laboratories. Schraders et al. [16] demonstrated similar findings with BCL2 expression in 5 of 18 (27.8%) putative BCL2(−) FLs when tested with alternate clones. All 5 of their cases demonstrated *BCL2* rearrangement as per FISH analyses.

We found no difference in BCL-XL or MCL1 expression between BCL2(−) and BCL2(+) FLs. BCL-XL and MCL1 are BCL2 family antiapoptotic proteins, which inhibit the proapoptotic proteins BAX and BAK. BCL-XL is expressed in many B-cell lymphomas and has been relevant in FL models in which induction of apoptosis was associated with decreased BCL-XL expression [3,4]. Overexpression of MCL1 has been described in both multiple myeloma and DLBCL and often is a consequence of 1q21 amplifications in the latter [19,20]. MCL1 has also been studied in t(14; 18)-positive FL cell lines, and when expressed, it can block B-cell receptor−induced apoptosis [5]. In addition, *MCL1*-transgenic mice develop FL-like lesions, similar to *IGH-BCL2* transgenic mice [6]. Overall, our findings suggest that BCL-XL overexpression is not a dominant factor in resistance to apoptosis in BCL2(−) FLs. Although not significant in our study, MCL1 expression in BCL2(−) FLs may warrant additional investigation to exclude a role for MCL1 in BCL2(−) FL, particularly because gene expression studies suggested MCL1 expression was elevated in such cases [12]. Overexpression of MCL1 in BCL2(−) FLs could be clinically relevant because small-molecule inhibitors of BCL2 family proteins (BH3 mimetics), including specific MCL1 inhibitors, are now becoming available for clinical trials and are relevant in lymphoma [21−23].

We observed significantly lower expression of BIM in BCL2(−) FLs than in BCL2(+) FLs, with both digital image analysis and with manual scoring. BIM is a BH3-only proapoptotic BCL2 family protein that functions as a potent activator of apoptosis through two mechanisms: direct activation of proapoptotic BAX and BAK proteins and/or neutralization of the antiapoptotic BCL2 proteins [24]. We postulate decreased BIM expression could represent an alternate mechanism leading to inhibition of apoptosis and may be relevant in FL for multiple reasons. First, BIM is one of the transcriptional targets of TAp73, a *TP73* gene isoform, located in the 1p36 region frequently deleted in FLs [25]. Hassan et al. [25] demonstrated significantly lower *BIM* gene expression in FLs than in

follicular hyperplasia, but there was no stratification based on BCL2 status. Second, FLs among Japanese women with a deletion site (2903 bp) polymorphism in intron 2 of *BIM* that abrogates its proapoptotic function had significantly worse progression-free survival than wild-type FL controls [26,27]. Third, in t(14; 18)-positive cell lines treated with venetoclax, we observed decreased expression of BIM as a potential resistance mechanism, whereby an antiapoptotic state is reestablished [8]. Finally, BIM plays a key role in the induction of apoptosis triggered by various chemotherapeutics, including paclitaxel, tyrosine kinase inhibitors, and bortezomib [28]. This may also be relevant in FL treatment as BIM is upregulated in FL cell lines after treatment with novel therapeutic agents [29]. Overall, the findings suggest decreased BIM expression may be relevant in establishing an antiapoptotic state in FLs lacking BCL2 overexpression.

Several studies have attempted to address the importance of the increased Ki-67 proliferative index in cytologically low-grade FLs [30,31]. However, it has not been studied systematically to our knowledge in BCL2(−) FL. In our study, the mean Ki-67 labeling index within follicles was 37.0% in BCL2(−) FLs compared with 43.2% in BCL2(+) FLs ($p = 0.177$). This seemed somewhat higher than expected. The high Ki-67 indices could be partially due to the significant number of grade 3 FLs in both groups (>30% of total cases). However, of the grade 1−2 BCL2(−) FLs, 3 of 11 (27.3%) had a Ki-67 proliferative index >30%, compared with 10 of 21 (47.6%) grade 1−2 BCL2(+) FLs. Although again somewhat higher than expected, we found no statistical difference between the two groups.

5. Conclusion

In summary, we describe a series of BCL2(−) FLs and examined key BCL2 family member expression. From a diagnostic standpoint, we remind readers that use of an alternate BCL2 antibody is advisable before making a diagnosis of BCL2(−) FL. Although the underlying pathogenic mechanisms in these BCL2(−) FLs remain to be fully elucidated, we demonstrated significantly decreased expression of BIM in BCL2(−) FLs, which we postulate could exert an overall antiapoptotic effect. The availability of specific BCL2 family inhibitors as targeted treatment makes this a promising area of future research. Indeed, epigenetic regulation of BIM expression is a strategy that has been explored in breast cancer and demonstrated to be relevant in lymphoid differentiation. In these studies, histone deacetylase inhibitors, hypomethylating agents, and EZH2 inhibitors (a recently approved strategy in refractory/relapsed FL) can upregulate BIM expression [24,32−34]. Our study provides rationale for exploring this in the setting of BCL2-negative FL.

Author statement

Daniel Socha: Writing- Original Draft, Data Curation, Visualization, Formal Analysis, Investigation. **Xiaoxian Zhao:** Formal Analysis, Investigation, Software. **Juraj Bodo:** Formal Analysis, Investigation. **Lisa Durkin:** Investigation. **Eric Hsi:** Conceptualization, Methodology, Supervision, Writing- Original Draft, Project administration, Resources.

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