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Original contribution

Proapoptotic protein BIM as a novel prognostic marker in mantle cell lymphoma☆,☆☆

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Summary Mantle cell lymphoma (MCL) is an aggressive B-cell lymphoma. Numerous studies have demonstrated many genetic aberrations in MCL in addition to the characteristic t(11:14), including frequent biallelic deletions of *Bim*, a proapoptotic member of the BCL-2 family. In mice, *Bim* deletion coupled with cyclin D1 overexpression generates pathologic and molecular features of human MCL. Since the regulation of apoptosis is crucial in MCL pathogenesis, we hypothesize that BIM expression may be associated with tumor cell survival. Clinical data and tissue from 100 nodal MCL cases between 1988 and 2009 were collected from three large academic medical centers. The average patient age of our MCL cohort was 65.5 years old (range, 42-97) with a 2:1 male to female ratio. Immunohistochemistry was performed with a validated anti-BIM antibody. Patients were separated into low and high BIM-expressing categories with a cutoff of 80%. As expected for a proapoptotic tumor suppressor, patients with high BIM expression were less likely to have progressive disease and more likely to have a complete response ($P = .022$). In addition, high BIM-expressing MCL tumors revealed a trend toward increased overall survival with this trend persisting in sub-analysis of Ann Arbor stages III and IV. No correlation between BIM expression, Ki-67 index, and MIPI score was observed, suggesting a role for BIM as a novel independent prognostic factor. While BIM is only one member of a complex family of apoptosis-regulating proteins, these findings may yield clinically relevant information for the prognosis and therapeutic susceptibility of MCL.

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1. Introduction

Mantle cell lymphoma (MCL) is a mature B-cell lymphoma accounting for 5% to 10% of non-Hodgkin lymphomas [1]. While subsets of patients display indolent disease, most patients have an aggressive disease course with a median overall survival of 3 to 5 years [1,2]. Clinically, most patients are diagnosed at an advanced stage (III-IV) with the median age of diagnosis between 65 and 70 years old [1,3]. Even with aggressive treatment and current therapies, most patients have rapid progression of their disease [4].

While MCL is classically characterized by the t(11:14)(q13;q32), leading to overproduction of cell cycle regulator Cyclin D1 facilitating G1 to S phase progression in an unchecked manner, further studies have elucidated numerous other genetic aberrations [5]. As earlier mice studies show that Cyclin D1 overexpression is not enough to recapitulate MCL, we now know that secondary genetic alterations and deregulated pathways are drivers of MCL progression in humans [6,7]. Additionally, genome wide studies display vast tumor heterogeneity with mutations in genes involved in DNA repair, histone modification, cell-cycle regulation, and apoptotic dysfunction, among others [7].

Apoptotic deregulation contributes to the pathogenesis of MCL. In cDNA microarray analysis, MCL exhibits alterations in the apoptotic cascade proteins, with an inclination toward anti-apoptosis [8]. Of the anti-apoptotic BCL-2 family proteins, BCL-2, BCL-X_L, and MCL-1 proteins are highly expressed in certain MCL lines [9-12]. Overexpression of BCL-X_L leads to constitutive nuclear factor-kB pathway activation, an important transcription factor involved in cell growth and survival [13]. Additionally, MCL-1 overexpression correlates with aggressive blastoid morphology, high proliferation rates, and p53 overexpression [14]. Alternatively, the proapoptotic protein BAX becomes inhibited through cytoplasmic sequestration due to Cyclin D1 expression [15]. Furthermore, the proapoptotic BH3-only protein BIM shows biallelic gene deletion in 5 of 12 MCL cell lines and loss of expression in 7 of 22 patients [16]. The homozygous deletion of *Bim* characterizes it as a novel tumor suppressor in MCL [17].

Creation of a mouse model with Cyclin D1 overexpression and deletion of proapoptotic *Bim* recapitulates the MCL phenotype, supporting BIM's role as a tumor suppressor and arguing for apoptotic deregulation as a factor in MCL lymphomagenesis [18]. In accordance with other hematopoietic and solid-organ malignancies, BIM's role as a tumor suppressor appears to have prognostic and therapeutic significance. For example, in chronic myeloid leukemia, *Bim* deletion polymorphisms lengthen the time for patients to achieve a major molecular response on tyrosine kinase inhibitors [19,20]. Additionally, loss of *Bim* or its expression in melanoma, colorectal cancer, intrahepatic cholangiocarcinoma, and EGFR-positive non-small cell lung carcinoma is associated with worse prognoses [21-25]. With the advent of targetable therapies acting on the apoptotic cascade, BIM may serve as a critical biomarker for emerging therapies such as small molecule, proapoptotic, BH3-mimetics, like venetoclax.

Since the clinical behavior of MCL is heterogeneous and regulation of apoptosis is a critical control point in MCL pathogenesis, we hypothesize that BIM expression may be associated with tumor cell survival. This study is the first to demonstrate BIM protein expression in human MCL, with clinical and histologic correlates.

2. Materials and methods

2.1. Study population

After institutional review board approval, 100 MCL patients with involved nodal disease from Yale-New Haven Hospital (YNH)(21), Brigham and Women's Hospital (BWH)(17), and University of Wisconsin Hospital and Clinics (WHC)(62) were selected. All tumors were positive for Cyclin D1 by immunohistochemistry (IHC). Portions of the WHC cohort also demonstrated t(11;14) by fluorescence in-situ hybridization (FISH). Among the 100 MCL patients identified, 16 patients were excluded due to insufficient tissue for histologic analysis. Patient data obtained via chart review consisted of clinical parameters such as Ann Arbor staging, Eastern Cooperative Oncology Group (ECOG) performance status, Mantle Cell Lymphoma International Prognostic Index (MIPI), transplant status, treatment modality, overall response rate (ORR), and dates of diagnosis, treatment, relapse, and death. Histopathologic parameters collected included the histologic variant (classic vs. blastoid) and Ki-67 proliferation index (Ki-67%). Blastoid variants were confirmed by WHO 2017 criteria including tumors that resemble lymphoblasts and have a high mitotic rate (>10–37.5 mitoses per 15 high-power fields).

2.2. Immunohistochemistry

Formalin-fixed paraffin-embedded involved nodal tissue from MCL patients was stained with the BIM rabbit monoclonal antibody clone C34C5 (Cell Signaling Technology, Danvers, MA). The antibody is reactive to the protein BIM isoforms EL, L, and S with exact epitopes proprietary. The commercial antibody is not FDA approved to our knowledge. Glass slides were deparaffinized by rehydrating with distilled water followed by heating to 95–102° Celsius at pH 9.0 for 30 minutes to induce epitope retrieval. Slides were cooled to room temperature, then placed in 3% H_2O_2 for 5 minutes, then incubated with BIM rabbit monoclonal antibody at 1:50 dilution for 30 minutes, then incubated in horseradish peroxidase (HRP) conjugated Donkey anti-Rabbit IgG antibody for 30 minutes, and finally incubated in diaminobenzidine (DAB) for 5 minutes. Hematoxylin counterstain (Avantik, Springfield, NJ) followed by dehydration and clearing in xylene was performed with a resin coverslip placed afterwards. As indicated by the manufacturer's recommendation, a wide

Table 1 Clinicopathologic characteristics of the MCL study population

	Total (%)	Low BIM (<80%)	High BIM (≥80%)
Study population	84 (100%)		
Males	55 (65.5%)	25 (45.5%)	30 (54.5%)
Females	29 (34.5%)	17 (58.6%)	12 (41.4%)
Ki-67% index	71 (100%)		
<30%	34 (47.9%)	26 (76.5%)	8 (23.5%)
≥30%	37 (52.1%)	24 (64.9%)	13 (35.1%)
MIPI score	66 (100%)		
<5.7	24 (36.4%)	10 (41.7%)	14 (58.3%)
5.7 ≤ score< 6.2	20 (30.3%)	13 (65%)	7 (35%)
≥6.2	22 (33.3%)	8 (36.4%)	14 (63.6%)
Histologic variant	84 (100%)		
Classic	71 (84.5%)	39 (54.9%)	32 (45.1%)
Blastoid	13 (15.5%)	3 (23.1%)	10 (76.9%)
Ann Arbor stage	71 (100%)		
I	8 (11.3%)	5 (62.5%)	3 (37.5%)
II	2 (2.8%)	1 (50%)	1 (50%)
III	14 (19.7%)	10 (71.4%)	4 (28.6%)
IV	47 (66.2%)	20 (42.6%)	27 (57.4%)
ECOG score	65 (100%)		
0	32 (49.2%)	19 (59.4%)	13 (40.6%)
1	21 (32.3%)	10 (47.6%)	11 (52.4%)
2	8 (12.3%)	1 (12.5%)	7 (87.5%)
3	2 (3.1%)	1 (50%)	1 (50%)
4	2 (3.1%)	2 (100%)	0 (0%)

range of controls were used to validate the antibody. For the MCLs from WHC, the same tissue microarray (TMA) was constructed that was used in the prior study by Oberley et al [26]. Patient tumors from BWH and YNH were separately embedded onto glass slides before staining.

2.3. Scoring

Analysis of the BIM IHC stained MCL nodal tissue was performed by two independent pathologists. On the TMA, multiple 0.6 mm cores from the same tumor were assessed and the highest BIM expression was quantified by percentage (0%-100%). On the whole tissue sections, the pathologists microscopically assessed multiple areas of BIM cytoplasmic protein staining, counting at 40x objective. Where possible, at least 3 nonadjacent fields were counted to result in total cells positive divided by total cells in aggregate. Cytoplasmic staining of any intensity was considered positive. Given the nature of this tumor type, the neoplastic tissues were fairly homogenous in their staining pattern. Due to the high inter-observer correlation of BIM expression (r = 0.97), the average expression percentages were used. Patients were separated into low and high BIM expressing categories with a cutoff of 80%, which corresponds to the median value of BIM expression percentages.

2.4. Statistics

Statistical analysis was conducted using SAS (version 9.4, SAS Inst Inc, Cary, NC) and Minitab (version 17, Minitab Inc, LLC, State College, PA) statistical analysis software. The association between BIM, a dichotomized variable, and the patients' clinicopathologic characteristics was evaluated using Chi-square test for categorical variables, two-sample-t-test for continuous variables, and log-rank test for survival data (Kaplan–Meier method). Utilizing BIM as a continuous variable, sensitivity analysis was conducted to confirm the results were consistent with the conclusion of significance. For this study, $P < .05$ (without multiple comparison adjustment) was considered significant.

3. Results

3.1. Patient demographics and clinicopathologic characteristics

In the study population, 65.5% (55/84) were male and 34.5% (29/84) were female with an approximate male to female ratio of 2:1. A larger proportion of men were high BIM expressers (54.5%, 30/55), while the majority of women tended to be low BIM expressers (58.6%, 17/29). The average Ki-67% was 27%, the median was 20%, and it ranged from 5% to 97%. There were 47.9% (34/71) of patients with Ki-67< 30% and 52.1% (37/71) with a Ki-67 ≥30%. For the MCL classic histologic variant, there were slightly lower than high BIM expressing tumors (54.9% vs. 45.1%, respectively). However, within the 13 blastoid histologic variant tumors, there was a larger percentage with high

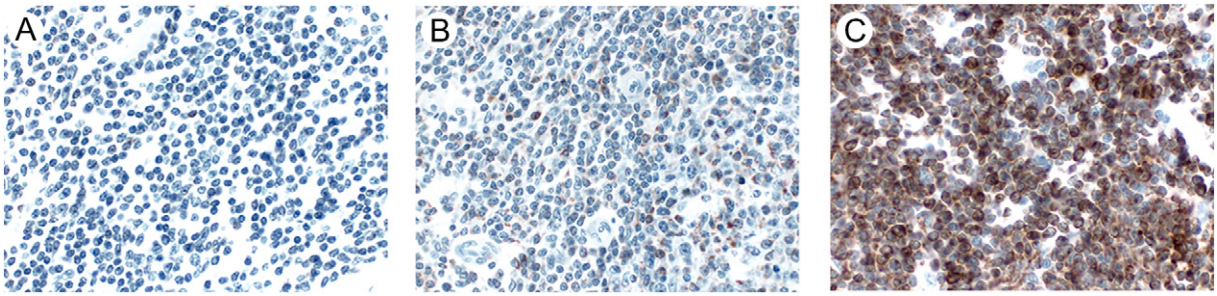

Fig. 1 Cytoplasmic expression of BIM protein in involved nodal MCL tissue. A, Low expression of BIM (~10%). B, Moderate expression of BIM (~60%). C, High expression of BIM (~95%).

versus low BIM expression (76.9% vs. 23.1%, respectively). The majority (85.9%) of patients were in Ann Arbor stages III and IV, with 19.7% (14/71) and 66.2% (47/71) in stages III and IV, respectively. The majority (81.5%) of patients had an ECOG score of 0 and 1. Patient dates of diagnosis ranged from 1988 to 2009. Patient ages at diagnosis ranged from 42 to 97 years old with an average age of 65.5 years old and a median age of 63 years old. Additional clinicopathologic characteristics are summarized in Table 1.

3.2. BIM IHC expression in MCL

BIM IHC staining displayed cytoplasmic protein expression in tumor cells (Fig. 1). The expression levels ranged from 5% to 100% with an average of 66% and median of 79.5%. Patients were evenly separated into low BIM (<80%) and high BIM (≥80%) expressing categories with 42 patients in each group (Fig. 2). Due to the high inter-observer correlation of BIM expression (r = 0.97), the expression percentages for each case were averaged.

3.3. MCL patient treatment interventions and overall response rate

Of the 68 patients with ORR data, 86.8% (59/68) received chemotherapy, 4.4% (3/68) received localized radiation therapy, 4.4% (3/68) received no intervention, 1.5% (1/68) had surgery (gastrectomy and splenectomy), 1.5% (1/68) received an unspecified form of therapy, and 1.5% (1/68) had no information available. Seventy-one percent (48/68) of patients received CHOP-based therapy (cyclophosphamide, doxorubicin, vincristine, and prednisone), while 55.9% (38/68) of patients received RCHOP-based therapy (addition of Rituximab). Rituximab alone or in combination with one or two other agents, such as fludarabine, bortezomib, cytarabine, or bendamustine comprised 7.4% (5/68) of patients. The rest of chemotherapy-treated patients received chlorambucil alone or in combination with one or two other agents (5.9%, 4/68), DICE therapy (dexamethasone, ifosfamide, cisplatin, etoposide) (1.5%, 1/68), and cyclophosphamide with radiation (1.5%, 1/68).

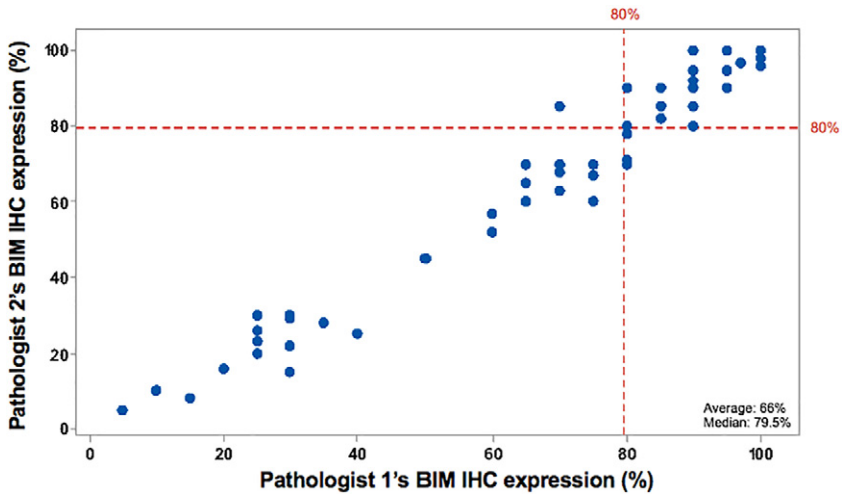

Fig. 2 High interobserver correlation (r = 0.97) was seen between both pathologists. A cutoff of 80% separates low and high BIM expressing MCL with an even distribution of patients in each group.

Table 2 Treatment interventions of MCL study population with BIM expression and ORR

Treatment Interventions	Total (%)	Low BIM (<80%)	High BIM (≥80%)	Complete response (CR)	Partial response (PR)	Stable disease (SD)	Progressive disease (PD)
	68 (100%)	30 (44.1%)	38 (55.9%)	-	-	-	-
RCHOP-based therapy	38 (55.9%)	13 (34.2%)	25 (65.8%)	25 (65.8%)	5 (13.2%)	1 (2.6%)	7 (18.4%)
CHOP-based therapy	10 (14.7%)	7 (70%)	3 (30%)	3 (30%)	0 (0%)	0 (0%)	7 (70%)
Rituximab alone + combination	5 (7.4%)	1 (20%)	4 (80%)	2 (40%)	1 (20%)	0 (0%)	2 (40%)
DICE therapy	1 (1.5%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
Chlorambucil alone + combination	4 (5.9%)	2 (50%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	4 (100%)
Cyclophosphamide + radiation therapy	1(1.5%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
Radiation therapy only (local)	3 (4.4%)	1 (33.3%)	2 (66.7%)	2 (66.7%)	0 (0%)	1 (33.3%)	0 (0%)
Surgery only [a]	1 (1.5%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
Treatment unspecified [b]	1 (1.5%)	0 (0%)	1 (100%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)
No intervention	3 (4.4%)	3 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (33.3%)	2 (66.7%)
Status unknown [c]	1 (1.5%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)

[a] Astrectomy and splenectomy.
[b] Patient was treated, but specific therapy was not documented.
[c] No information available on treatment.

Of patients treated with RCHOP-based therapy, 65.8% (25/38) had a complete response (CR), 13.2% (5/38) had a partial response (PR), 2.6% (1/38) had stable disease (SD), and 18.4% (7/38) had progressive disease (PD). In patients treated with CHOP-based therapy without Rituximab, 30% (3/10) had a CR and 70% (7/10) had PD. In patients treated with rituximab alone or in combination with one or two other agents, 40% (2/5) had a CR, 20% (1/5) had a PR, and 40% (2/5) had PD. The rest of the patients, whose therapies included unconventional chemotherapy, surgery, radiation, or no intervention, were more likely to have PD than CR (66.7%, vs. 20%, respectively).

Of patients treated with RCHOP-based therapy, 65.8% (25/38) had high BIM expressing tumors and 34.2% (13/38) had low BIM expressing tumors. Of patients treated with just CHOP-based therapy (no Rituximab), 30% (3/10) had high BIM expressing tumors and 70% (7/10) had low BIM expressing tumors. Of patients treated with Rituximab alone or in combination with other agents, 80% (4/5) had high BIM expressing tumors and 20% (1/5) had low BIM expressing tumors. See Table 2 for additional details.

3.4. Patients with high BIM (≥80%) expressing MCL are less likely to have progressive disease and more likely to have a complete response

In patients with tumors demonstrating high BIM expression, 56.3% (18/32) and 15.6% (5/32) had a CR and PR, respectively (Table 3). In contrast, patients with tumors demonstrating low BIM expression had a CR of 41.7% (15/36) and a PR of 2.8% (1/36). Additionally, 6.3% (2/32) of patients with high BIM expressing tumors achieved SD while only 2.8% (1/36) of patients with low BIM expressing tumors did so. For patients with PD, 52.7% (19/36) had low BIM expressing tumors while only 21.8% (7/32) had high BIM expressing tumors. These overall differences were statistically significant using a χ^2 test ($P = .022$).

3.5. Patients with high BIM expressing MCL tumors reveal a trend toward increased overall survival

A total of 59 MCL patients had survival data for analysis. At 2 years, both high and low BIM expressing cohorts had similar overall survival probabilities at 70% (Fig. 3). At 5 years, low BIM expressing cohorts had an overall survival probability of 47%, while high BIM expressing cohorts were at 56%. At 10 years, low BIM expressing cohorts had an overall survival probability of 30%, while high BIM expressing cohorts were at 45%. This trend continues to widen with time, but shows no statistical significance ($P = .33$). In subanalysis of Ann Arbor stage, high BIM expressing tumors showed a better overall survival trend than low BIM expressing tumors in stages III and IV, but not in stages I and II (Fig. 4). Subanalysis of only classic variant MCL tumors (removing all blastoid variant tumors) and patients who re-

Table 3 Overall response rate of low vs. high BIM expressing MCL [a]

ORR	Low BIM IHC expression (<80%)	High BIM IHC expression (≥80%)
Complete response	41.7% (15/36)	56.3% (18/32)
Partial response	2.8% (1/36)	15.6% (5/32)
Stable disease	2.8% (1/36)	6.3% (2/32)
Progressive disease	52.7% (19/36)	21.8% (7/32)

[a] $P = .022$.

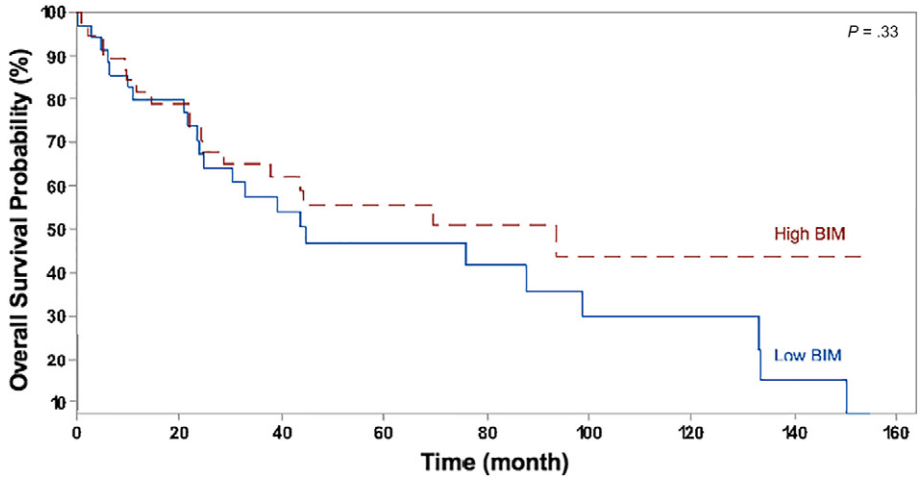

Fig. 3 Kaplan–Meier survival curve comparing high BIM (red) and low BIM expressing (blue) MCL.

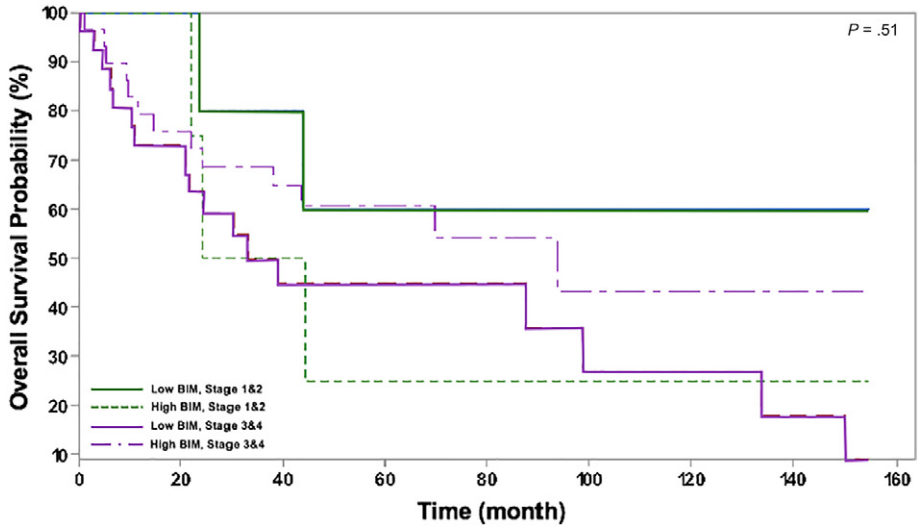

Fig. 4 Kaplan–Meier survival curve comparing high and low BIM tumor expression in Ann Arbor stages 3 and 4 (purple) and Ann Arbor stages 1 and 2 (green).

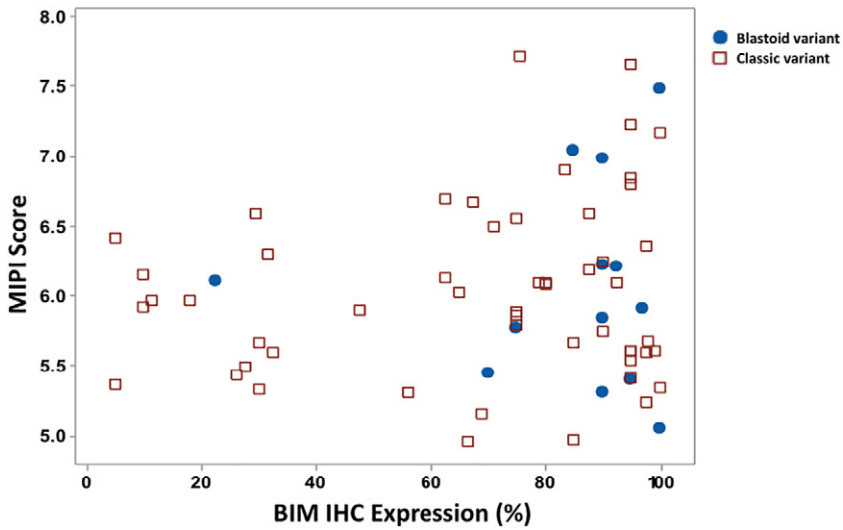

Fig. 5 Scatterplot of MCL BIM IHC expression(%) vs. MIPI score. Blastoid variant tumors are represented as blue dots. Classic variant tumors are represented as red squares.

ceived only RCHOP-related therapies showed a similar overall survival trend between high and low BIM MCL expressing tumors. Additional progression free survival analysis was performed and showed no trend or statistically significant results.

3.6. No correlation between BIM IHC expression, Ki-67 proliferation index, and MIPI scores

There was no correlation between tumor BIM IHC expression levels and MIPI scores (Fig. 5). Patients with high BIM expressing tumors had a wide range of MIPI scores ranging from 4.96 to 7.71. Patients with low BIM expressing tumors also had a wide range of MIPI scores ranging from 4.97 to 7.81. Patients with low risk MIPI scores (<5.7) had BIM expression levels ranging from 5 to 100%. Patients with intermediate risk MIPI scores (5.7 ≤ score< 6.2) had BIM expression levels ranging from 10 to 97%. Patients with high risk MIPI scores (≥6.2) had BIM expression levels ranging from 5 to 100%. Between the two morphologic categories, 15.5% (13/84) of the patient cohort consisted of blastoid morphologic variant tumors while 84.5% (71/84) consisted of classic variant tumors. In the blastoid variant group, 76.9% (10/13) of the tumors were in the high BIM expressing category, while 23.1% (3/13) were in the low BIM expressing category, which is significantly skewed to high BIM compared to the classic MCL cohort (P = .006). Additionally, 30.8% (4/13) of blastoid variant tumors were in the low risk MIPI score group, 38.5% (5/13) in the intermediate risk MIPI group, and 30.8% (4/13) in the high risk MIPI group.

There was no correlation between tumor BIM IHC expression levels and Ki-67% (Fig. 6). Patients with high BIM expressing tumors had Ki-67% ranging from 2 to 97%. Patients with low BIM expressing tumors had Ki-67% ranging from 5 to 80%. Patients with Ki-67% <30% had BIM expression levels ranging from 5 to 100%. Patients with Ki-67% ≥30% had BIM expression levels ranging from 10 to 100%. Of the blastoid variants, 54% (7/13) of tumors had a Ki-67% <30%, while 46% (6/13) of tumors had a Ki-67% ≥30%. The average Ki-67% of the blastoid variant MCL tumors was 40.1% and of the classic variant MCL tumors was 24.3%.

4. Discussion

Given the promising data on the utility of BIM as a biomarker in various tumors, our study is the first to explore its expression profile for MCL in the context of clinicopathologic correlates. To combat the clinical heterogeneity of MCL, the development of prognostic factors to predict patient outcomes and aid treatment algorithms is essential. As such, the advent of Ki-67 proliferation index, MIPI score, and morphologic subtyping has assisted in these endeavors [4,27,28]. Recent research highlights numerous other prognostic factors. For example, TP53 mutations in younger patient cohorts are associated with poorer outcomes with conventional therapies, suggesting that these patients should be considered for frontline trials of novel agents [29]. Likewise, MCLs that lack SOX11 expression are categorized as more indolent, arguing for "watch and wait" protocols as initial management [30-34]. Serving as a novel molecular biomarker, the MCL35 assay, which contains a 17-gene proliferation signature, defines patients with significantly different overall survivals independent of MIPI scores [35]. It's possible that a combination of biomarkers is necessary for optimal outcome predictions. Therefore, further characterizing BIM's role in human MCL may lead to better clinical management and may help identify advantageous novel agents targeting the apoptotic cascade.

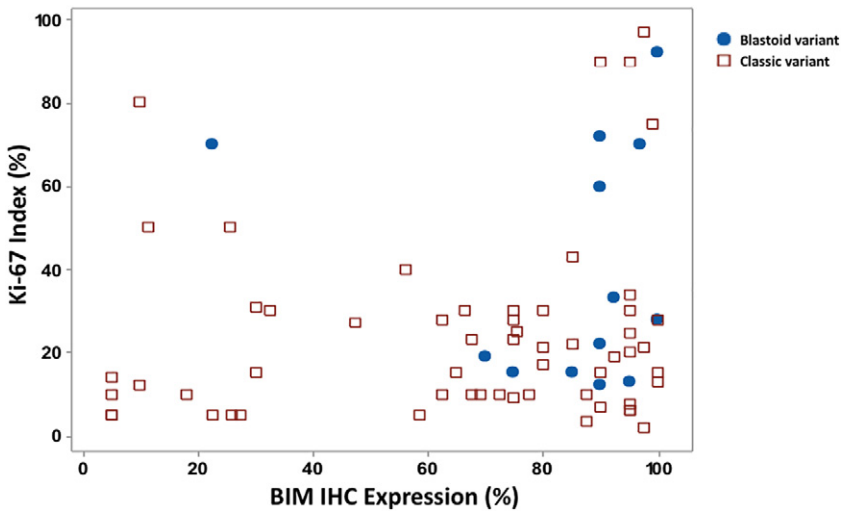

Fig. 6 Scatterplot of MCL BIM IHC expression(%) vs. Ki-67 proliferation index(%). Blastoid variant tumors are represented as blue dots. Classic variant tumors are represented as red squares.

In this study, BIM expression is strongly correlated with the patient's ORR; high BIM expressers are more likely to have a CR and less likely to have PD. A complete response of meaningful magnitude and duration is associated with longer OS [36]. The majority of patients are treated with RCHOP-based chemotherapies, which is still the most prevalent treatment regimen for MCL patients currently [37]. Similar to how outcomes have improved considerably for younger cohorts treated with upfront aggressive therapies [38,39], clinicians may consider similar strategies for patients with regard to BIM expression. In support of this paradigm, tumor BIM expression and its association with therapy response is seen in chronic myeloid leukemia, multiple myeloma, and follicular lymphoma where BIM deficiency results in poorer response to therapy through defective apoptotic signaling mechanisms [40-42]. Although chemotherapy history is relatively thorough in this study, bone marrow stem cell transplant data is incomplete. Consolidation therapy with autologous stem cell transplant (ASCT) demonstrates improved progression free survival and achievement of molecular remission [43-45]. Moreover, allogeneic stem cell transplant is considered potentially curative [46]. This limitation was primarily a result of obtaining truly long term outcome, where older records were often incomplete or missing. In addition, large academic centers tend to serve as referral centers where patients receive second opinions but may not continue their care there. Having this data in future, larger datasets may allow investigation into post-transplant response rates of different BIM expressers.

Our survival analysis yields a trend toward better overall survival for high BIM expressers. Although not statistically significant, possibly due to limited study power, the trend seems promising for BIM as a prognostic marker in MCL. Subanalysis of just the classic variant MCL cohort, higher Ann Arbor stage disease, and patients who received only RCHOP-related therapy displays the same overall survival trend. As seen in patients with melanoma, reduced BIM expression is associated with poor 5-year overall survival [21]. In patients with colorectal cancer receiving 5-fluorouracil-based adjuvant chemotherapy, elevated BIM expression results in better disease-free survival and increased overall survival [22]. In patients with intrahepatic cholangiocarcinoma, elevated BIM expression is associated with increased overall survival and decreased lymph node metastases [23]. In epidermal growth factor receptor-tyrosine kinase inhibitor treated NSCLC patients with *Bim* deletion polymorphisms, there is decreased overall survival and post-recurrence survival [47]. These studies and ours highlight the potential for prognostic impact of BIM expression in tumors.

There is no correlation between MCL BIM expression and Ki-67% and MIPI score. Although the Ki-67% of intrahepatic cholangiocarcinoma is inversely associated with BIM expression, our findings in MCL do not recapture this phenomenon [23]. Genes and pathways responsible for both apoptosis and cellular proliferation are complex. For instance, the tumor suppressor p53 regulates genes responsible for cell death by inhibiting anti-apoptotic proteins BCL-2, BCL-X_L, and MCL-1 and activating proapoptotic proteins BAX and BAK, a function similar to the BH3-only proapoptotic protein BIM [48-50]. Also, p53 regulates cell proliferation by inducing expression of p21, leading to inhibition of cyclin dependent kinases, and triggering G1 arrest [51]. Moreover, prior studies have reported a Ki-67% of ≥30% in 16% and >30% in 30% of MCL patients [52,53], and a median Ki-67% of 20%. [54]. While our study shows a similar median Ki-67%, the proportion of tumors with Ki-67% ≥30% (52.1%) is higher than reported. This is because 16 MCLs with Ki-67% but no associated BIM IHC was excluded. If all 87 MCLs with Ki-67% values were included, the tumors with Ki-67% ≥30% would be 29%, which is within the range of prior studies. Additionally, there is no correlation between MCL BIM expression and MIPI score. The MIPI score, a clinical prognostic factor based on age, leukocyte count, lactate dehydrogenase levels, and ECOG score, facilitates treatment protocols and helps predict survival [55,56]. Due to the heterogeneity of clinical parameters and complexity of molecular pathways, an association between MCL BIM expression with Ki-67% and MIPI score is unclear. These results may further support BIM's role as an independent prognostic factor.

In the 13 cases of blastoid variant MCL in our study, the majority (76.9%) have high BIM expression. While blastoid variants typically harbor complex karyotypes and TP53 mutations [57-60], with subsets containing *c-myc* gene translocations [61], research evaluating alterations of the apoptotic cascade in this variant are limited. It is possible that blastoid variant tumors develop without input from genes involved in apoptosis. Another reason may be that intrinsic apoptotic machinery is up-regulated to combat the aggressive phenotype of this variant. While p53 expression is associated with increased BIM levels due to release from anti-apoptotic proteins MCL-1, BCL-2, or BCL-X_L [62], most TP53 mutations result in loss of function thereby abrogating this effect. Mice with MYC overexpression promote apoptosis by up-regulating proapoptotic BIM and suppressing anti-apoptotic BCL-2 expression, thus lowering the apoptotic threshold [63]. As blastoid variant MCL is notable for MYC overexpression [64], this may explain the increased BIM expression noted in this population. Furthermore, while MYC-induced apoptosis utilizes the p53 pathway, MYC can also induce apoptosis via a p53-independent pathway through BIM [63]. Thus, mice with *Bim* deletions must rely on the p53 pathway for apoptosis [63]. In theory, MCL patients with TP53 mutations may need to up-regulate BIM expression in order to undergo apoptosis, and those with TP53 mutations and low BIM expression may have a dismal prognosis. As an area of further investigation, determining the TP53 and *Myc* mutational status of these aggressive variant tumors may be of interest.

A limitation of this study is the clinical and therapeutic diversity of the three cohorts. The WHC cohort comprised a larger majority (54.8%) of patients who were younger

(average age 63 years), diagnosed more recently (median year 2006), and mainly received RCHOP-based chemotherapies. Additionally, a portion of this cohort received Bortezomib, in a combination chemotherapy called VcR-CVAD (Bortezomib, Rituximab, Cyclophosphamide, Vincristine, Doxorubicin, and Dexamethasone). In contrast, the BWH and YNH cohorts were older (average age 67 years), diagnosed earlier (median year 2000), and had varied treatment regimens with less frequency of Rituximab therapy. The BWH and YNH cohorts had a higher proportion of progressive disease compared to the WHC cohort. Subsequent individual survival analysis of each cohort shows no trends or statistically significant results. Not surprisingly, treatment cohorts with Rituximab [65] and younger and fitter MCL patients [39] tend to have better outcomes. Another limitation of this study is the lack of patient molecular and cytogenetic data. While most MCLs are characterized by the t(11;14), a subset are negative for Cyclin D1 overexpression and t (11;14) [66]. Instead, some of these tumors harbor chromosomal rearrangements of CCND2 [66]. While the entire cohort had positive Cyclin D1 IHC expression, only a subset received cytogenetic analysis. Finally, our study only analyzes a single protein among a myriad of other effectors within the apoptotic cascade. Further studies to understand the complex interactions among these proteins is needed.

MCL has benefitted from advances in molecular techniques and the subsequent emergence of targeted therapies. In particular, ibrutinib, an inhibitor of Bruton's tyrosine kinase, an important protein in B-cell antigen receptor signaling [67], has been approved for relapsed/refractory MCL [68]. Also, the proteasome inhibitor bortezomib, which was initially approved for relapsed MCL, has shown increased progression free survival when integrated with traditional therapies, and is now being used as a frontline drug [69]. Similarly, immunomodulatory agent lenalidomide, together with Rituximab, has been recently studied as an initial treatment option for MCL patients [70]. Gaining interest in MCL, the BCL-2 inhibitor venetoclax, which functions to displace proapoptotic BH3-only proteins from BCL-2 [71], has achieved promising results in relapsed/refractory MCL [72]. Hence, the development of biomarkers targeting the apoptotic cascade could dictate clinical management of patients taking these novel drugs.

In conclusion, this novel clinicopathologic study is the first to investigate BIM expression in human MCL and its relationship to patient outcomes and response to therapy. Our results show that MCL with high levels of proapoptotic BIM expression are more likely to result in a patient's complete response rather than progressive disease following therapy. Likewise, the increased overall survival of high BIM expressers over time is promising as a prognostic factor. Finally, the lack of correlation of BIM expression with other established prognostic factors such as MIPI and Ki-67 index gives it weight as an independent prognostic factor. Further investigation into BIM and its association with therapies targeting the apoptotic cascade may reveal additional information regarding its role as a biomarker in MCL, or other solid and hematopoietic tumors alike.

Author contribution

MLX, SGK and JDW initiated the project, proposed and executed the experiments and were involved in all aspects of data interpretation as well as manuscript preparation and editing. EAM and DTY contributed cases and were involved in manuscript preparation and editing. XP carried out statistical analysis and was involved in manuscript preparation and editing.

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