SJIF Impact Factor: 5.464

WORLD JOURNAL OF ADVANCE HEALTHCARE RESEARCH

ISSN: 2457-0400
Volume: 5.
Issue: 6.
Page N. 268-281
Year: 2021

Research Article www.wjahr.com

PROGNOSTIC SIGNIFICANCE OF BCL-2 AND BCL-6 IMMUNOSTAINING IN B-CELL NON-HODGKIN'S LYMPHOMA

Reem Kafa, MD[1]*, Firas Hussein, MD[2] and Ali Daoud, MD[3]

[1]Resident, Department of Hematology, Tishreen University Hospital, Latakia, Syria.
[2]Assistant Professor, Department of Hematology, Tishreen University Hospital, Latakia, Syria.
[3]Assistant Professor, Department of Pathology, Tishreen University Hospital, Latakia, Syria.

Received date: 21 October 2021 **Revised date:** 11 November 2021 **Accepted date:** 01 December 2021

***Corresponding Author: Dr. Reem Kafa, MD**

Resident, Department of Hematology, Tishreen University Hospital, Latakia, Syria.

ABSTRACT

Purpose: The aim of this study was to identify a possible effect of Bcl-2 and Bcl-6 proteins on the clinical behavior and outcome in different subtypes of B-cell non-Hodgkin lymphoma (B-NHL). **Methods:** We retrospectively studied Bcl-2 and Bcl-6 expression by immunohistochemistry (IHC) in 145 patients with diffuse large B-cell lymphoma (DLBCL), 86 patients with follicular lymphoma (FL), and 53 patients with mantle cell lymphoma (MCL), and analyzed their prognostic relevance and effect on treatment response in these cohorts. **Results:** Positive Bcl-2 expression related strongly to lower rates of complete response (CR) after first-line treatment with Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) in both DLBCL ($p=0.001$) and MCL ($p=0.01$). Bcl-2 was recognized as an independent predictor of poor overall survival (OS) (HR=2.9, $p<0.001$, and HR=3.7, $p= 0.02$ in DLBCL and MCL, respectively). Patients with positive expression of Bcl-6 were more likely to achieve CR after R-CHOP in both DLBCL ($p=0.006$) and FL ($p=0.012$). Bcl-6 positive expression was an independent indicator of a favorable OS (HR=0.3, $p<0.001$, and HR=0.4, $p=0.04$ in DLBCL and FL, respectively). An IHC score based on the expression of Bcl-2 and Bcl-6 in DLBCL accurately defined three risk groups with markedly different OS ($p<0.001$). This new score outweighed the International Prognostic Index (IPI) as a prognostic indicator (HR=3.2 vs 2.2, $p<0.001$). **Conclusion:** Bcl-2 and Bcl-6 protein expression detected by IHC can be reliably used to help predict treatment response and survival trends in a wide subset of B-NHLs.

KEYWORDS: B-cell non Hodgkin lymphoma (B-NHL), Bcl-2, Bcl-6, diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), prognosis.

INTRODUCTION

B-cell non Hodgkin lymphomas (B-NHLs) represent a heterogeneous group of hematologic malignancies[1,2] and while their prognosis is mainly dependent on histopathology,[3] the prognosis for each histologic variant is known to be related to multiple differences in tumor biomarkers, and is influenced by well-recognized clinical features summarized in the International Prognostic Index (IPI) such as age, the Ann-Arbor stage, and the performance status.[4] In fact, IPI has been widely used as the main tool for estimating overall survival after standard of care treatment.[5] Nonetheless, independent of IPI, patients can still experience early treatment failure in response to the standard combination of Rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP),[4] suggesting the existence of additional oncogenic events that are responsible for chemoresistance.[6] Several studies have focused on the specific role of cellular markers detectable by immunohistochemistry (IHC) and their link to different aspects of tumor biology,[7] with conflicting results concerning a number of these markers.

Bcl-2 (B-cell lymphoma -2) is an anti-apoptotic protein. It was originally discovered because of its involvement in translocation *t*(14;18) in follicular lymphoma (FL).[8] Later on, it was found that the aberrant expression of Bcl-2 contributes to the pathogenesis of many types of human malignancies, including leukemias, lymphomas, and solid cancers independent of translocation t(14;18).[1] Deregulation of the *BCL-2* gene can arise from

substantially different genetic abnormalities in distinct lymphoma subtypes, causing Bcl-2 protein overexpression and impaired apoptosis, especially in response to chemotherapy. Venetoclax, a highly selective Bcl-2 inhibitor, has shown promising results in several subtypes of lymphomas with high expression of Bcl-2.[6] While various reports have linked Bcl-2 protein overexpression to dismal outcome in diffuse large B-cell lymphoma (DLBCL),[4,9-13] other reports failed to show similar results.[14,15] Also, data about the prognostic impact of Bcl-2 and its effect on response to chemotherapy in other lymphomas such as mantle cell lymphoma (MCL) are still lacking.

Bcl-6 (B- cell lymphoma -6) is a transcriptional repressor protein encoded by the *BCL-6* proto-oncogene. Its expression is largely restricted to germinal center (GC)-B-cells where it is required for the formation of the GC, facilitation of somatic hypermutation, and the T-cell mediated immune response.[16,17] In lymphoid malignancies, in addition to the constitutive *BCL-6* expression of GC B-cells, the gene can be deregulated by different mechanisms, causing Bcl-6 overexpression, which in turn causes GC expansion and promotes survival of GC-derived B-cell lymphomas.[18,19] Gene aberrations concerning the *BCL-6* proto-oncogene and their effect on lymphoma outcome have been widely studied, and discussing avenues for the therapeutic targeting of Bcl-6 have gained critical importance. Yet, data on associating Bcl-6 expression on a protein level with the response to standard treatment modalities and outcome of certain B-cell lymphomas remain conflicting.

The aim of this study was to analyze the protein expression of BCl-2 and Bcl-6 by IHC in three common B-NHLs; DLBCL, FL, and MCL, to investigate the effect of these two biomarkers on response to the conventional R-CHOP, and to define whether they have an independent value for predicting survival in these lymphomas.

PATIENTS AND METHODS

Patient selection

We retrospectively examined the medical records of patients who were diagnosed with a B-NHL and managed at Tishreen University Hospital's (TUH) Cancer Center during the years 2006-2015. The last follow-up data were obtained in January, 2021. 284 patients including 145 patients with DLBCL, 86 patients with FL, and 53 patients with MCL were enrolled in this study based on the availability of clinical information and histologic material for definite diagnosis. All lymphoma specimens were reviewed using morphologic and immunohistochemical criteria according to the WHO classification of malignant lymphomas.

We excluded patients who were <18 years old, patients with an active concurrent malignancy, and patients whose biopsy showed composite or unclassifiable histology. Primary mediastinal lymphomas and cases of DLBCL and FL with primary extranodal presentation were also excluded from the analysis to guarantee maximum uniformity of the patient cohort.

Clinical information regarding patient characteristics, staging, therapies, and best overall response, as assessed by treating physicians, were extracted from record review and patient charts. All patients were previously untreated and received 4-8 cycles of standard CHOP or CHOP-like chemotherapy regimen in combination with rituximab ± radiotherapy as first-line therapy. Response to therapy was assessed at the end of induction and complete response (CR) was defined as the disappearance of all physical and radiographic evidence of lymphoma for at least four weeks after systemic chemotherapy and/ or radiation.

This study was approved by the local ethics committee.

Immunohistochemistry

Histologic specimens for all 284 patients were available. Five-micrometer sections from each formalin-fixed, paraffin-embedded block were cut, pre-treated, and then stained with antibodies to Bcl-2 (Rabbit monoclonal antibodies, clone EP36, Bio SB) and Bcl-6 (Rabbit monoclonal antibodies, clone RBT-bcl6, Bio SB). Previously known positive cases for both Bcl-2 and Bcl-6 were used as an external control in order to evaluate immunoreactivity. Immunostains were considered positive if 30% or more of the tumor cells were stained by the antibodies.

Statistical analysis

Frequencies and means were compared using the chi-square test and the one-way Anova test. Overall survival (OS) time was calculated from the date of diagnosis until death or date of last follow-up, while progression-free survival (PFS) time was calculated from diagnosis to disease progression or death from any cause. Survival curves were estimated by the Kaplan-Meier analysis, using the log-rank test to analyze the statistical differences between the groups. Multivariate regression analysis of PFS and OS was carried out according to the Cox model to test the variables analyzed in the study as potential independent prognostic factors. $P<0.05$ was considered to indicate statistical significance. Statistical analysis was performed using Microsoft Excel and SPSS version 26.

RESULTS

Bcl-2 and Bcl-6 in DLBCL

145 patients diagnosed with primary nodal DLBCL were included in this study, their major characteristics are shown in **table 1**. Median age of presentation ranged between 18 and 85 years, with a median of 54 years.

Median follow-up time was 60 months (range, 8-129 months). Overall, CR was achieved by 65.5% of patients and was strongly correlated with IPI risk stratification (low:83%, low- intermediate: 64%, high-intermediate: 50%, high:30%, *p<0.001*).

Bcl-2 overexpression was detected in 89 patients, and these patients were more likely to present at an advanced stage (*p=0.043*), and to have B symptoms (*p=0.006*) and high LDH levels (*p=0.007*) at diagnosis. Bcl-2 positive patients were less likely to achieve CR after first-line R-CHOP compared to Bcl-2 negative patients (55% vs 82%, *p=0.001*).

Bcl-6 was positive in 63 patients. There was no significant differences in clinical parameters between Bcl-6 positive and Bcl-6 negative patients. On the other hand, CR was markedly higher in patients with overexpression of Bcl-6 compared to those with negative Bcl-6 expression (78% vs 56%, *p=0.006*).

Both median PFS and OS were not reached by the end of our study. 5-year PFS was 41%, while 5-year OS was 46%. 5-y OS according to IPI groups was 74% in the low risk group, 32% in the low-intermediate group, 21% in the high-intermediate group, and 9% in the high risk group, (*p<0.001*).

Survival analysis using the Kaplan-Meier model showed unfavorable trends in Bcl-2 positive patients compared to Bcl-2 negative patients with regard to both PFS (*p=0.001*) and OS (*p<0.001*) **Figure 1 (A, B)**. On the other hand, a clear correlation between Bcl-6 positive expression and better PFS and OS was observed (*p=0.003* for PFS, and *p<0.001* for OS) **Figure 1 (C, D)**.

Multivariate Cox regression analysis of OS showed that Bcl-2 expression was a strong indicator for poor prognosis (HR=2.9, *p<0.001*), while Bcl-6 expression predicted a favorable outcome (HR=0.3, *p<0.001*). Both were independent of IPI (HR=2.2, *p<0.001*).

In an attempt to test whether the expression status of both Bcl-2 and Bcl-6 can be used to accurately categorize our patients into more defined risk groups than IPI, we designed a score system where a point was given to each histologic variable that predicted a negative prognosis according to Cox regression (Bcl-2 positivity and Bcl-6 negativity). The sum of negative factors resulted in a risk score that ranged from zero to two in each case. When analyzing survival according to the Kaplan-Meier method, a significant difference in overall survival was evident between the first group (23 patients with Bcl-2^+ /Bcl-6^- status, score: zero), the second group (33 patients with Bcl-2^-/Bcl-6^- status and 40 patients with Bcl-2^+/Bcl-6^+ status, score: one), and the third group (49 patients with Bcl-2^+/Bcl-6^- status, score: two) (*p<0.001*). **Figure 1(E, F).** In fact, 5-y OS was 87% in the first (favorable) group, 51% in the second (intermediate) group, and 22% in the third (unfavorable) group. In multivariate analysis, this IHC-score outweighed IPI as an independent predictor of patient outcome (HR=3.2 vs. HR=2.2, *p<0.001*).

Bcl-2 and Bcl-6 in FL

Table 2 summarizes the main characteristics of 86 FL patients who were included in this study. Median age at presentation was 55.5 years (range; 27-81 years). Median follow-up was 110 months (range; 14-170 months). Patients were assigned to three groups based on their Follicular Lymphoma International Prognostic Index (FLIPI) scores: low risk (33 patients), intermediate risk (16 patients), and high risk (37 patients). A significant correlation between FLIPI risk stratification and CR after first-line therapy was evident as CR rates for these groups were 88%, 75%, and 49%, respectively, (*p=0.002*).

8 patients had low expression of Bcl-2. All but one were diagnosed at an early stage, had normal LDH levels at presentation, and achieved CR after first-line therapy. Of note, all Bcl2^- patients had grade 3 histology. Due to its small size, we did not compare clinical or survival data of the Bcl-2^- group to those of the Bcl-2^+ group.

Bcl-6 was positive in 9 patients. These patients had less number of involved nodes (*p<0.001*), a smaller chance of bone marrow infiltration (*p=0.03*), and were more likely to present at an early stage (*p=0.001*). CR rates were higher among Bcl-6^+ patients compared to Bcl-6^- patients (80% vs 54%, *p=0.012*).

While median PFS and OS were not reached for the low and intermediate- risk groups by the end of our study, they recorded 42 months and 61 months, respectively, in the high-risk group. 5 y- PFS and 5 y-OS in the entire cohort were 58% and 71%, respectively. 5-y OS rates in each of the FLIPI prognostic groups were as follows: 91% in the low risk group, 75% in the intermediate risk group, and 51% in the high risk group *(p<0.001).*

Figure 2 depicts the relationship between Bcl-6 status and PFS (**A**) and OS (**B**) according to the Kaplan-Meier survival analysis. While Bcl-6 positivity correlated to a clearly better OS (*p=0.007*), it did not have a similar effect on PFS (*p=0.1*).

Using multivariate analysis, only FLIPI risk score maintained a predictive value of PFS (HR=2.1, *p=0.001*), while both FLIPI risk score and Bcl-6 positivity were independent predictors of OS (HR=2.2, *p=0.01* and HR=0.4, *p=0.04*, respectively), with Bcl-6 positive expression predicting a favorable outcome.

Bcl-2 and Bcl-6 in MCL

53 patients with MCL were included in this study, their data are listed in **table 3**. Age at diagnosis ranged

between 36 and 77 years, with a median of 60 years. Patients were followed for a median time of 69 months (range; 22-107 months). Using the Mantle cell lymphoma International Prognostic Index (MIPI) risk score, patients were assigned to three categories: low-risk (28 patients), intermediate-risk (9 patients), and high-risk (16 patients). 62% of our study cohort were able to achieve CR after first-line therapy. CR was correlated to the MIPI score (*p=0.023*), with rates of 79%, 56%, and 38% in the low, intermediate, and high risk groups, respectively.

Bcl-2 expression was positive in 28 patients. These patients were more likely to present at an advanced stage of their disease (*p=0.02*) and to have B symptoms (*p=0.02*). Bcl-2 positivity showed no association to the other clinical parameters including the MIPI risk score. CR, however, was associated to Bcl-2 status (*p=0.02*) with rates of 80% in the Bcl-2$^-$ group and 46% in the Bcl-2$^+$ group.

6 patients showed aberrant expression of Bcl-6, and while all of these patients presented with the classical variant, Bcl-6 expression failed to show a correlation to any of the studied clinical parameters including CR.

5-y PFS was 51% in the entire cohort, with a median PFS of 50 months and 35 months in the MIPI intermediate risk group and high risk group, respectively. 62% of MCL patients were still alive at 5 years, with a median OS of 62 months in the intermediate risk group and 44 months in the high risk group. Both median PFS and median OS were not reached in the low risk group by the end of the study. 5-y OS rates were 82%, 56% and 31% in the low, intermediate, and high-risk groups, respectively (*p<0.001*).

Bcl-2 positive patients had significantly worse PFS (*p=0.002*) and OS (*p=0.003*) compared to Bcl-2 negative patients, as shown by survival curves according to Kaplan-Meier analysis. **Figure3**. Multivariate analysis proved that Bcl-2 positive expression was an independent and a stronger factor than the MIPI score in predicting overall survival in MCL (HR= 3.7, *p=0.02* vs HR=2.3, *p=0.003*, respectively).

DISCUSSION

Bcl-2 anti-apoptotic protein is the most well studied member of the Bcl-2 family, which tightly regulates the cellular program of apoptosis through the balanced effects of protein-protein interactions. While preserving the integrity of the mitochondrial outer membrane with its embedded hydrophobic domain, Bcl-2 also inactivates several pro-apoptotic proteins such as BAK and BAX which would otherwise oligomerize and release several apoptogenic molecules from the mitochondrion. Bcl-2 protein overexpression consequentially leads to apoptosis evasion, and that plays a crucial role in lymphoma pathogenesis, progression, and drug resistance.[1,8,20]

In concordance with earlier studies,[10,15,21,22] Bcl-2 positive expression correlated to worse PFS and OS in our cohort of DLBCL, and this adverse impact on survival was independent of IPI. Patients with positive Bcl-2 protein expression were also more likely to present with adverse clinical factors such as advanced stage, B symptoms, and higher LDH levels. Only 55% of Bcl-2 positive patients were able to achieve a complete response after first-line therapy. In-vitro studies on murine and human leukemia cell lines have demonstrated that, in Bcl-2 transfected cells, chemotherapy induced arrest of proliferation, but unlike in control cells, death by apoptosis was prevented, and drug withdrawal resulted in reinitiation of cell growth.[23,24] This explains why lymphoma cells with high Bcl-2 expression exhibit higher resistance to multiple antineoplastic agents.

Both Kucukzeybek et al and Perves et al found no significant difference in OS between DLBCL patients with negative or weakly positive Bcl-2 and high Bcl-2 expressions.[14,25] While these different results observed may be because of methodological variations, Iqbal et al suggested that the existing controversy regarding Bcl-2 expression and survival may be related to studying DLBCL as a single entity.[13] In fact, *BCL-2* is upregulated by different mechanisms in the germinal center B-cell-like (GCB) and activated B-cell-like (ABC) subgroups of DLBCL, and that may have distinct clinical implications depending on the cell of origin. In the GCB subgroup, in a very similar fashion to FL, Bcl-2 expression is mainly a result of the t(14;18).[26] Alternatively, *BCL-2* upregulation in ABC-like DLBCL may be mediated through the nuclear factor kappa-B (NF-κB) pathway. Another possible mechanism is the amplification of the chromosomal locus 18q21 on which *BCL-2* resides.[27,28] In Iqbal et al study, Bcl-2 expression was related to worse survival profiles only in ABC-like DLBCL.

Similarly to ABC DLBCL, *BCL-2* amplification is frequently found in MCL, while the translocations are rare.[29] Another cytogenetic abnormality contributing to high Bcl-2 protein expression in MCL is deletion of 13q14 locus that contains genes that negatively regulate *BCL-2* at the posttranscriptional level.[30] 53% of MCL patients in our present study had positive Bcl-2 expression, and this group had markedly worse PFS and OS. In fact, Bcl-2 protein expression and the MIPI score were the only independent predictors of OS. The fact that high Bcl-2 expression was not correlated with the level of LDH in MCL suggests that the poor outcome is mainly due to delayed cell death or resistance to treatment, but not to increased cell proliferation.[9] It is well known that MCLs respond poorly to chemotherapy and that could be related to the negative expression of BAX, a pro-apoptotic protein seen in a majority of MCLs. Bcl-2 binds and inactivates BAX, further reducing its cellular levels.[31] Indeed, patients with negative Bcl-2 expression were almost twice likely to

experience full remission after first-line therapy compared to Bcl-2 positive patients in the present study. The results of this study support the use of Bcl-2 protein expression as a potential indicator for the use of an aggressive treatment approach such as autologous stem cell rescue after attaining first remission and possibly the front-line use of anti-Bcl-2 targeted therapy.

Although translocation *t*(14;18)(q32;q21) is considered the genetic hallmark of FL, about 10% FL cases lack this *BCL-2* gene translocation, and these display distinct molecular features with activated B cell-like, NF-κB and proliferation expression profiles and frequent lack of Bcl-2 protein expression,[1,32] 8 out of 86 patients diagnosed with FL in this study had low levels of Bcl-2, and despite having grade 3 histology, all but one presented at an early stage. All Bcl-2 negative patients maintained a PFS of at least 87 months and all were still alive by the end of follow-up. Of note, 7 of these 8 patients had high Bcl-6 expression by IHC, a finding similar to a study by Takeshita et al, where a tendency for Bcl-6 translocations and high expression of Bcl-6 protein was found more frequently in Bcl-2 negative patients[33] Bcl-6 expression was found to be inversely related to p53 overexpression, which in turn has been related to a worse survival profile in lymphoma patients.[34,35]

Bcl-6 protein expression was positive in 43% of DLBCL cases and 57% of FL cases in the current study. Bcl-6 is almost universally expressed in GC-derived B-cell lymphomas including DLBCLs and FLs.[18] The relatively low percentage of Bcl-6 positivity in our study may be explained by technical factors related to staining, interpretation, and scoring of positive results.[16,36-38] Our study agreed with a number of reports showing a better survival trend among DLBCL patients with positive Bcl-6 expression.[7,39,40] On the other hand, in a clinical trial by Winter et al, OS of patients with Bcl-6 expression who were treated with R-CHOP was not statistically different from that of Bcl-6 negative patients.[16] In our study, all patients were treated with first-line R-CHOP and yet, Bcl-6 positive status related significantly to better PFS and OS. Patients who overexpressed Bcl-6 were also more likely to obtain CR after R-CHOP. These results prove that Bcl-6 positivity is indeed a strong prognostic marker in DLBCL patients treated with R-CHOP. This contradiction of results could be because of the different length of the follow-up period; median follow-up in our study was 5 years compared to a median of 3.4 years in Winter's. By combining the expression status of Bcl-6 and Bcl-2 in DLBCL patients, we were able to devise a cumulative score where Bcl-2 positivity and Bcl-6 negativity served as risk factors. This IHC-score enabled us to categorize DLBCL patients into three defined prognostic groups (favorable, intermediate, and unfavorable) that had significant differences with regard to overall survival. The new score proved to have a stronger prognostic value than IPI in clinical risk assessment in DLBCL when the two were compared using multivariate analysis.

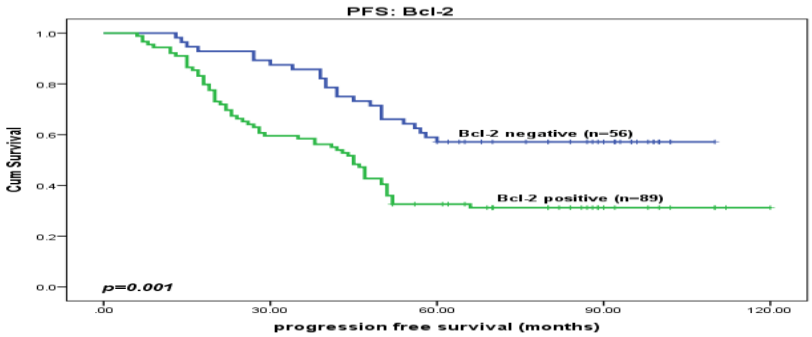
Bcl-6 overexpression in FL was related to a number of favorable characteristics, such as less involvement of nodal areas, absence of bone marrow infiltration, and presenting at an early stage. FL patients who were Bcl-6 positive also had better CR rates after first-line R-CHOP in our study. Although Bcl-6 overexpression failed to yield a statistically significant correlation with PFS, it was, beside the FLIPI score, the only independent predictor of OS. There is a paucity of data on the effect of Bcl-6 expression on the response to chemotherapy in FL, but a study by Bilalovic et al had somewhat similar results to our study, where patients with high levels of Bcl-6 expression had favorable OS and time to treatment failure (TTF).[36] Bcl-6 protein expression and its favorable impact on survival in lymphoma is considered quite controversial. Expression of Bcl-6 is pivotal for the maintenance of the GC reactions; it favors the sustained proliferation of B-cells involved in GC formation and suppresses the transcription of the p53 tumor suppressor gene in GC B-cells allowing the DNA breaks necessary for somatic hypermutation and immunoglobulin class switch recombination. Therefore, the constitutive expression of Bcl-6 as a result of translocation or mutation deregulating the *BCL-6* proto-oncogene may contribute to lymphagenesis through maturation arrest and a pathologic expansion of GC cells. Based on that, Bcl-6 expression, analogous to Bcl-2 expression, can be expected to be a poor prognostic factor.[16,36,41] The best evidence we found to be explanatory of the favorable outcome seen in patients with positive Bcl-6 expression in our series is that the neoplastic GC cells retain the high susceptibility to apoptosis found in their normal counterparts, and therefore, they are more sensitive to chemotherapy.[19,42]

Lastly, 6 out of a total of 53 MCL patients (11%) in the present study had an aberrant expression of Bcl-6 protein by IHC, and that agrees with the fact that MCL cells derive from naïve pre-germinal center and mantle-zone B lymphocytes and do not classically express GC markers. Bcl-6 positivity was neither related to a specific clinical feature, nor it had a prognostic value in these patients. The aberrant expression of Bcl-6 in MCL can be a result of chromosomal alterations involving the *BCL-6* gene such as translocation or an extra copy of the gene. It has also been demonstrated that 15-30% of MCL cases carry somatic mutations of the immunoglobulin heavy chain variable genes (*IGVH*), implying that the neoplastic cells in these cases have been exposed to the GC microenvironment.[17,43,44]

In conclusion, our study has demonstrated that Bcl-2 protein expression held a strong and an independent negative impact on response to chemotherapy and survival in both DLBCL and MCL, while Bcl-6 protein expression was associated with lower rates of

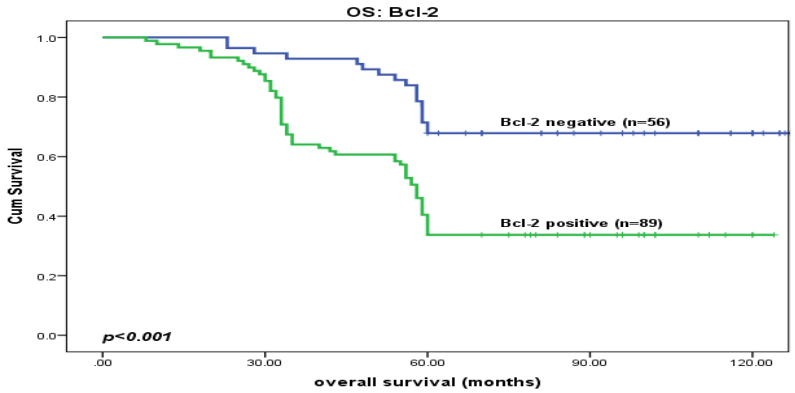
chemoresistance and longer survival in both DLBCL and FL. Detecting these cellular markers by IHC is fast and readily available and can be used as a reliable means to improve risk stratification of lymphoma patients and plan the best treatment strategy accordingly.

Figures and figure legends



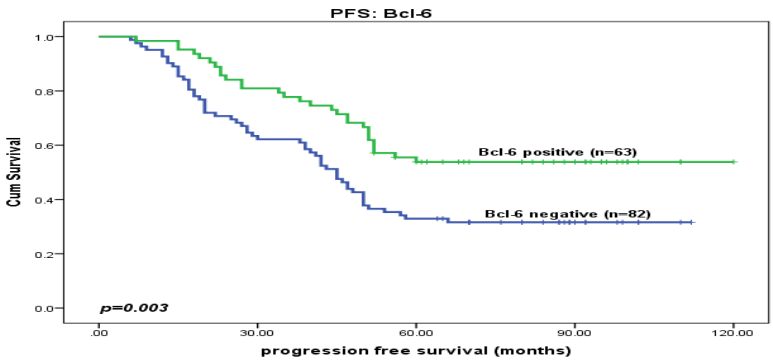
Number at risk					
Bcl-2 negative	56	50	33	17	0
Bcl-2 positive	89	53	27	9	1

Figure 1: A Progression-free survival (PFS) of 145 diffuse large B-cell lymphoma (DLBCL) patients according to Bcl-2 protein expression. Bcl-2 positive expression was associated with worse PFS (*p=0.001*).



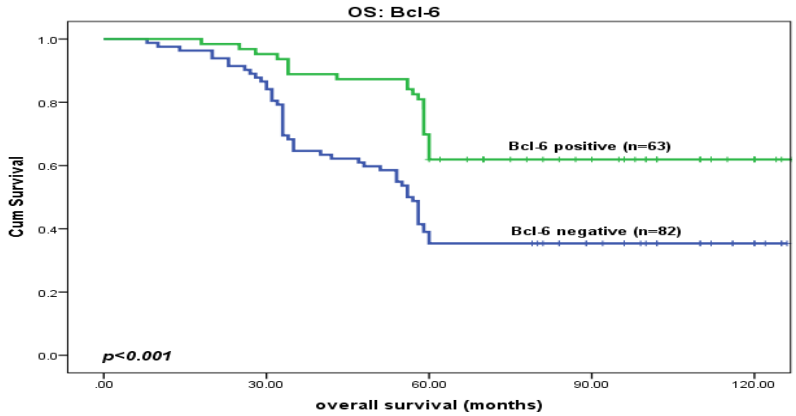
Number at risk					
Bcl-2 negative	56	53	40	26	14
Bcl-2 positive	89	78	36	22	3

Figure 1 B: Overall survival (OS) of 145 diffuse large B-cell lymphoma (DLBCL) patients according to Bcl-2 protein expression. Bcl-2 positive expression was associated with worse OS (*p<0.001*).



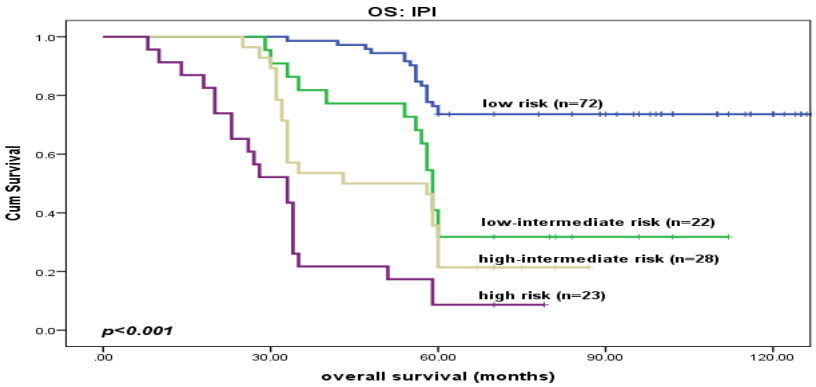
Number at risk					
Bcl-6 negative	82	52	27	10	0
Bcl-6 positive	63	51	33	18	1

Figure 1 C: Progression-free survival (PFS) of 145 diffuse large B-cell lymphoma (DLBCL) patients according to Bcl-6 protein expression. Bcl-6 positive expression was associated with better PFS (*p=0.003*).



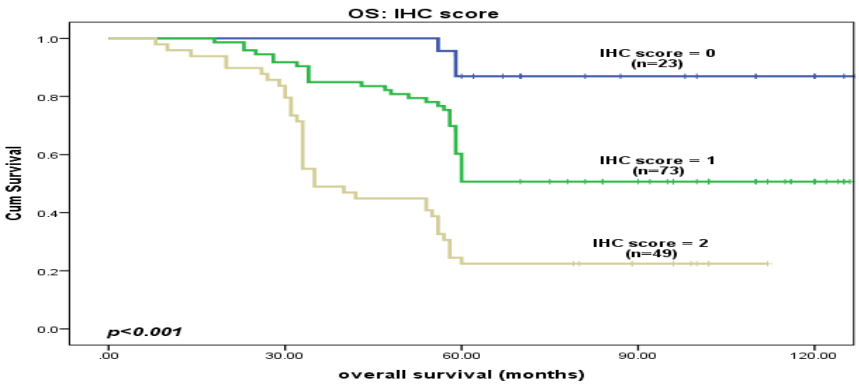
Number at risk					
Bcl-6 negative	82	71	32	22	7
Bcl-6 positive	63	60	44	26	10

Figure 1 D: Overall survival (OS) of 145 diffuse large B-cell lymphoma (DLBCL) patients according to Bcl-6 protein expression. Bcl-6 positive expression was associated with better OS (*p<0.001*).



Number at risk					
Low	72	72	55	45	17
Low-intermediate	22	21	9	3	0
High-intermediate	28	26	10	0	0
High	23	12	2	0	0

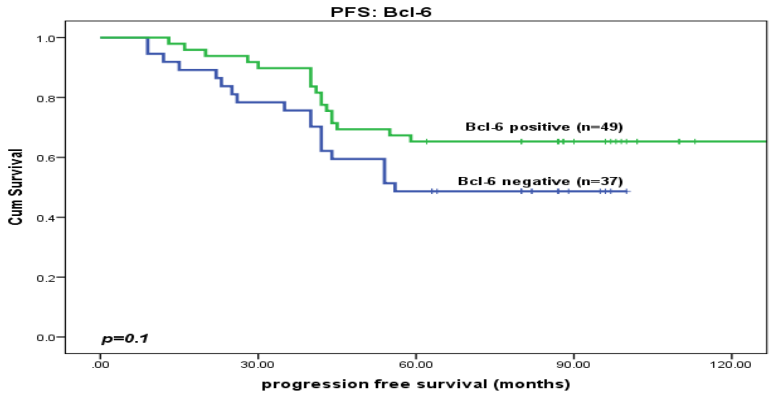
Figure 1 E: Overall survival (OS) of 145 diffuse large B-cell lymphoma (DLBCL) patients according to the International Prognostic Index (IPI). IPI accurately defines low and high risk patients (*p<0.001*). However, Kaplan-Meier curves depict no significant difference between the intermediate risk groups and the high risk group with regard to OS.



Number at risk					
0	23	23	20	11	7
1	73	67	44	30	10

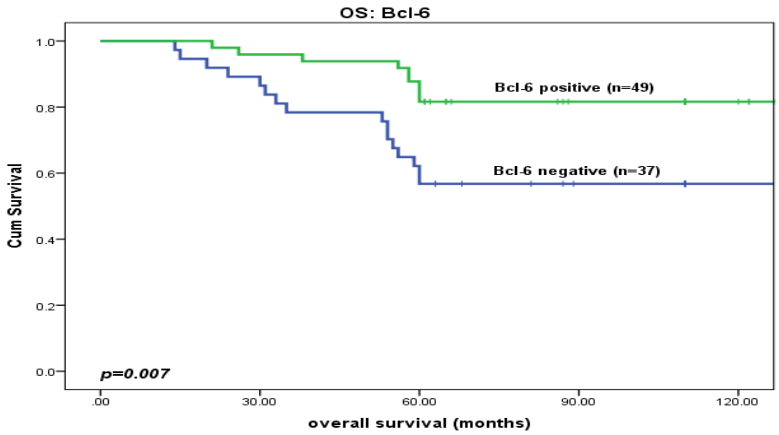
2 49 41 12 7 0

Figure 1 F: Overall survival (OS) of 145 diffuse large B-cell lymphoma (DLBCL) patients according to our devised Immunohistochemistry score (IHC score) based on the expression status of Bcl-2 and Bcl-6 in each case. IHC score successfully categorized DLBCL patients into three defined risk groups with significant differences in OS between cases without risk factors (score=0), cases with one risk factor (score=1), and cases with two risk factors (score=2), (*p<0.001*).



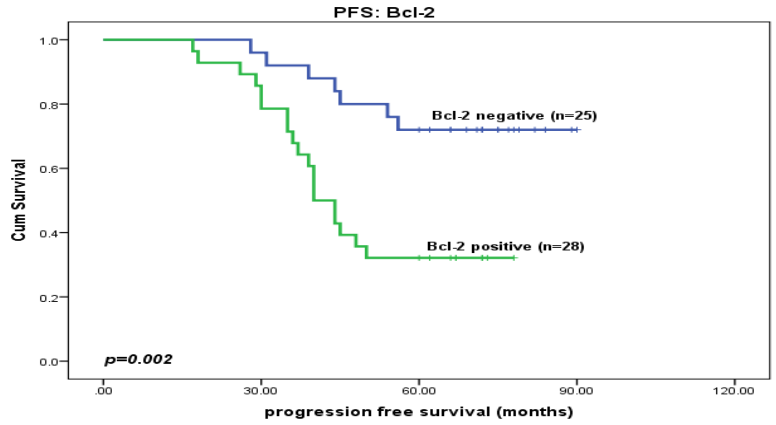
Number at risk
Bcl-6 negative 37 29 18 4 0
Bcl-6 positive 49 45 32 16 1

Figure 2 A: Progression-free survival (PFS) of 86 follicular lymphoma (FL) patients according to Bcl-6 protein expression. Bcl-6 positive expression had no effect on PFS (*p=0.1*).



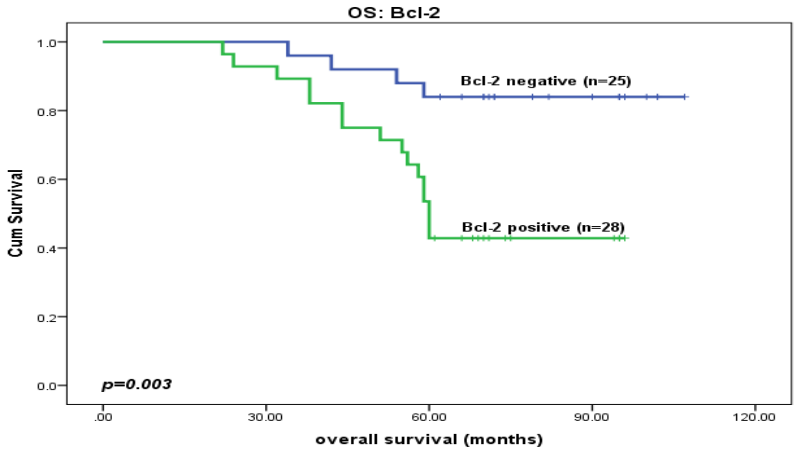
Number at risk
Bcl-6 negative 37 33 23 15 5
Bcl-6 positive 49 47 43 30 18

Figure 2 B: Overall survival (OS) of 86 follicular lymphoma (FL) patients according to Bcl-6 protein expression. Bcl-6 positive expression was associated with better OS (*p=0.007*).



Number at risk					
Bcl-2 negative	25	24	18	1	0
Bcl-2 positive	28	24	9	0	0

Figure 3 A: Progression-free survival (PFS) of 53 mantle cell lymphoma (MCL) patients according to Bcl-2 protein expression. Bcl-2 positive expression was associated with worse PFS (*p=0.002*).



Number at risk					
Bcl-2 negative	25	25	21	11	0
Bcl-2 positive	28	26	15	4	0

Figure 3 B: Overall survival (OS) of 53 mantle cell lymphoma (MCL) patients according to Bcl-2 protein expression. Bcl-2 positive expression was associated with worse OS (*p=0.003*).

Tables

Table 1: DLBCL patient characteristics according to Bcl-2 and Bcl-6 protein expression.

Parameter	Bcl-2 status (n=145)		Log-rank	Bcl-6 status (n=145)		Log-rank
	Negative (n=56)	Positive (n=89)		Negative (n=82)	Positive (n=63)	
Gender						
Male	25	44		37	32	
Female	31	45	*P=0.6*	45	31	*P=0.5*
Age (y)						
60 or less	39	60		55	44	
More than 60	17	29	*P=0.8*	27	19	*P=0.7*
ECOG PS						
Good (0-1)	38	47		47	38	
Poor (2 or more)	18	42	*P=0.07*	35	25	*P=0.7*

B symptoms Absent Present	27 29	23 66	***P=0.006***	24 58	26 37	*P=0.1*
Ann- Arbor stage Early (I-II) Advanced (III-IV)	31 25	34 55	***P=0.04***	35 47	30 33	*P=0.6*
No. of extranodal sites 0-1 2 or more	50 6	85 4	*P=0.1*	76 6	59 4	*P=0.8*
LDH Normal Elevated	38 18	40 49	***P=0.007***	44 38	34 29	*P=0.9*
IPI risk group Low Low-intermediate High-intermediate High	34 7 8 7	38 15 20 16	*P=0.2*	41 12 13 16	31 10 15 7	*P=0.4*
CR	82%	55%	***P=0.001***	56%	78%	***P=0.006***
5-y PFS	57%	31%	***P=0.002***	32%	54%	***P=0.007***
5-y OS	68%	34%	***P<0.001***	35%	62%	***P=0.002***

ECOG: European Cooperative Oncology Group; PS: performance status; LDH: lactate dehydrogenase; IPI: International Prognostic Index; CR: complete response (after first-line treatment); PFS: progression-free survival; OS: overall survival.

Table 2: FL patient characteristics according to Bcl-6 protein expression.

Parameter	**Bcl-6 status (n=86)**		**Log-rank**
	Negative (n=37)	**Positive (n=49)**	
Gender Male Female	22 15	25 24	*P=0.43*
Age (y) 60 or less More than 60	24 13	34 15	*P=0.65*
Histologic grade 1 2 3	1 11 25	17 21 11	***P<0.001***
B symptoms Absent Present	19 18	33 16	*P=0.13*
Ann- Arbor stage Early (I-II)	5 32	24 25	***P=0.001***

Advanced (III-IV)	18	43	
	19	6	***P<0.001***
No. of nodal areas			
4 or less	28	45	
More than 4	9	4	***P=0.03***
BM infiltration			
Absent	15	24	
present	22	25	*P=0.43*
Hemoglobin (g/dL)	17	32	
12 or more	20	17	*P=0.07*
Less than 12			
LDH	9	24	
Normal	8	8	
Elevated	20	17	*P=0.06*
FLIPI risk group	54%	80%	***P=0.01***
Low	49%	65%	*P=0.12*
Intermediate			
High	57%	82%	***P=0.01***
CR			
5-y PFS			
5-y OS			

FL: follicular lymphoma; BM: bone marrow; LDH: lactate dehydrogenase; FLIPI: Follicular Lymphoma-specific International Prognostic Index; CR: complete response (after first-line treatment); PFS: progression-free survival; OS: overall survival.

Table 3: MCL patient characteristics according to Bcl-2 protein expression.

Parameter	**Bcl-2 status (n=53)**		**Log-rank**
	Negative (n=25)	**Positive (n=28)**	
Gender			
Male	16	19	
Female	9	9	*P=0.8*
Age (y)			
60 or less	13	12	
More than 60	12	16	*P=0.5*
ECOG PS			
Good (0 or 1)	17	13	
Poor (2 or more)	8	15	*P=0.1*
B symptoms			
Absent	15	8	
Present	10	20	***P=0.02***
Ann- Arbor stage			

Early (I-II) Advanced (III-IV)	14 11	7 21	***P=0.02***
MCL form Classical Leukemic	21 4	22 6	*P=0.6*
Bulky nodes Absent present	18 7	17 11	*P=0.4*
LDH Normal Elevated	13 12	12 16	*P=0.5*
MIPI risk group Low Intermediate High	17 2 6	11 7 10	*P=0.08*
CR	80%	46%	***P=0.01***
5-y PFS	72%	32%	***P=0.004***
5-y OS	84%	43%	***P=0.002***

MCL: mantle cell lymphoma; ECOG: Eastern Cooperative Oncology Group; PS: performance status; LDH: lactate dehydrogenase, MIPI: Mantle cell lymphoma International Prognostic Index; CR: complete response (after first-line treatment); PFS: progression-free survival; OS: overall survival.

Authorship

Contribution: R.K. conceived and designed the study, analyzed the data, and wrote the manuscript. F.H. contributed patients, provided clinical information, and supervised data analysis. A.D. performed the pathological review. F.H. and A.D. both helped writing the manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

REFERENCES

1. Klanova, M; Klener, P. BCL-2 Proteins in Pathogenesis and Therapy of B-Cell Non-Hodgkin Lymphomas. *Cancers*, 2020; *12*: 938.
2. Swerdlow, S.H.; Campo, E.; Pileri, S.A.; Harris, N.L.; Stein, H.; Siebert, R.; Advani, R.; Ghielmini, M.; Salles, G.A.; Zelenetz, A.D.; et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*, 2016; *127*: 2375–2390.
3. Freedman, A; Friedberg, J. Pretreatment evaluation, satging, and response assessment of non-Hodgkin lymphoma. Rosmarin, A (Ed.), *UpToDate*. Retrieved, 2020; 1: 2021, from https://www.uptodate.com/contents/pretreatment-evaluation-staging-and-response-assessment-of-non-hodgkin-lymphoma/contributor-disclosure.
4. Thieblemont, C; Briere, J. MYC, BCL2, BCL6 in DLBCL: impact for clinics in the future? BLOOD, 2013; 21: 121, 12.
5. Sakopta, S; Shaikh, H. Non-Hodgkin Lymphoma. [Updated 2020 Dec 14]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK559328/?report=calssic.
6. Batlevi, C; Sha, F; Alperovich, A; Ni, A; Smith, K; Ying, Z; Soumerai, J; Caron, P; Falchi, L; Hamilton, A; Hamlin, P; Horwitz, S; Joffe, E; Kumar, A; Matasar, M; Moskowitz, C; Noy, A; Owens, C; Palomba, L; Straus, D; Von Keudell, G; Zelenetz, A; Seshan, V; Younes, A. Follicular lymphoma in the modern era: survival, treatment outcomes, and identification of high-risk subgroups. Blood Cancer Journal, 2020; 10: 74.
7. Chung, KM; Chang, ST; Huang, WT; Lu, CL; Wu, HC; Hwang, WS; Chang, KY; Chuang SS. Bcl-6 expression and lactate dehydrogenase level predict prognosis of primary gastric diffuse large B-cell lymphoma. *Journal of the Formosan Medical Association*, 2013; 112: 382e389.
8. Tzifi, F; Economopoulou, C; Gourgiotis, D; Ardavanis, A; Papageorgiou, S; Scorilas, A. The

Role of BCL2 Family of Apoptosis Regulator Proteins in Acute and Chronic Leukemias. *Advances in Hematology*, 2012, Article ID 524308.
9. Hermine, O; Haioun, C; Lepage, E; d'Agay, MF; Briere, J; Lavignac, C; Fillet, G; Salles, G; Marolleau, JP; Diebold, J; Reyes, F; Gaulard, P. for the Group d'Etude des Lymphomes de I'Adulte (GELA). Prognostic Significance of bcl-2 Protein Expression in Aggressive Non-Hodgkin's Lymphoma. *Blood*, 1996; 87(1): l, 265-27.
10. Mahmoud, H; El-Sakhawy, Y. Significance of Bcl-2 and Bcl-6 immunostaining in B-Non Hodgkin's lymphoma. *Hematology Reports*, 2011; 3: e26.
11. Barrans, S; Carter, I; Owen, R; Davies, F; Patmore, R; Haynes, A; Morgan, G; Jack, A. Germinal center phenotype and bcl-2 expression combined with the International Prognostic Index improves patient risk stratification in diffuse large B-cell lymphoma. *BLOOD*, 2002; 15: 99 - 4.
12. Neto, A; Siqueira, S; Dulley, F; Chauobah, A; Belesso, M; Saboia, R; Ruiz, M; Chamone, D; Pereira, J. Bcl-2 protein frequency in patients with high-risk diffuse large B-cell lymphoma. *Sao Paulo Med J*, 2010; 128(1): 14-7
13. Iqbal, J; Neppali, V; Wright, G; Dave, B; Horsman, D; Rosenwald, A; Lynch, J; Hans, C; Weisenburger, D; Greiner, T; Gascoyne, R; Campo, E; Ott, G; Muller-Hermelink, HK; Delabie, J; Jaffe, E; Grogan, T; Conners, J; Vose, J; Armitage, J; Staudt, L; Chan, W. *BCL2* Expression Is a Prognostic Marker for the Activated B-Cell–Like Type of Diffuse Large B-Cell Lymphoma. *Journal of Clinical Oncology*, 2006; 24: 6 – 20.
14. Kucukzeybek, B; Bener, S; Calli, A; Pakosy, T; Payzin, B. Prognostic Signifcance of Bcl-2 and p53 Protein Expressions and Ki67 Proliferative Index in Diffuse Large B-cell Lymphoma. *Turk J Hematol*, 2013; 30: 275-282.
15. Colomo L, López-Guillermo A, Perales M, Rives S, Martínez A, Bosch F, Colomer D, Falini B, Montserrat E, Campo E. Clinical impact of the differentiation profle assessed by immunophenotyping in patients with diffuse large B-cell lymphoma. *Blood*, 2003; 101: 78-84.
16. Winter, J; Horing, S; Krajewska, M; Variakojis, D; Habermann, T; Fisher, R; Kurtin, P; Macon, W; Chhanabhai, M; Feglar, R; His, E; Medeiros, J; Weick, J; Reed, J; Gascoyne, R. Prognostic significance of Bcl-6 protein expression in DLBCL treated with CHOP or R-CHOP:a prospective correlative study. *BLOOD*, 2006; 1: 107 - 11.
17. Gualco, G; Weiss, L; Harrington, W; Bacchi, C. BCL6, MUM1 AND CD10 EXPRESSION IN MANTLE CELL LYMPHOMA. *Appl Immunohistochem Mol Morphol*, 2010; 18(2): 103–108.
18. Bunting, K; Melnick, A. New effector functions and regulatory mechanisms of BCL6 in normal and malignant lymphocytes. *Curr Opin Immunol*, 2013; 25(3): 339–346.
19. Yang, H; Green, M. Epigenetic Programing of B-Cell Lymphoma by BCL6 and Its Genetic Deregulation. *Front. Cell Dev. Biol*, 2019; *7: 272.*
20. Klasa, R.J.; Gillum, A.M.; Klem, R.E.; Frankel, S.R. Oblimersen Bcl-2 antisense: Facilitating apoptosis in anticancer treatment. *Antisense Nucleic Acid Drug Dev*, 2002; *12*: 193–213.
21. Maartense E, Kramer MH, le Cessie S, Kluin-Nelemans JC, Kluin PM, Snijder S, Noordijk EM. Lack of prognostic signifcance of BCL2 and p53 protein overexpression in elderly patients with diffuse large B-cell non-Hodgkin's lymphoma: results from a population-based non-Hodgkin's lymphoma registry. *Leuk Lymphoma*, 2004; 45: 101-107.
22. Biasoli I, Morais JC, Scheliga A, Milito CB, Romano S, Land M, Pulcheri W, Spector N. CD10 and Bcl-2 expression combined with the International Prognostic Index can identify subgroups of patients with diffuse large-cell lymphoma with very good or very poor prognoses. *Histopathology*, 2005; 46: 328-333.
23. Miyashita T, Reed JC: Bcl-2 gene transfer increases relative resistance of S49.1 and WEH17.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticosteroids and multiple chemotherapeutic drugs. *Cancer Res*, 1992; 52: 5407.
24. Reed JC, Kitada S, Takayama S, Miyashita T: Regulation of chemoresistance by the bcl-2 oncoprotein in non-Hodgkin's lymphoma and lymphocytic leukemia cell lines. *Ann Oncol*, 1994; 5: 61.
25. Pervez S, Nasir MI, Moatter T, Ahsan A, Haq A, Siddiqui T. Characterization of genetic lesions in apoptosis-regulating and proliferation control genes in diffuse large B-cell nonHodgkin's lymphoma. *J Cancer Res Ther*, 2009; 5: 254-262.
26. Bolen, C.R.; Klanova, M.; Trneny, M.; Sehn, L.H.; He, J.; Tong, J.; Paulson, J.N.; Kim, E.; Vitolo, U.; Di Rocco, A.; et al. Prognostic impact of somatic mutations in diffuse large B-cell lymphoma and relationship to cell-of-origin: Data from the phase III GOYA study. *Haematologica*, 2019.
27. Davis RE, Brown KD, Siebenlist U, et al: Constitutive nuclear factor kappa B activity is required for survival of ctivated B cell-like diffuse large B cell lymphoma cells. *J Exp Med*, 2001; 194: 1861-1874.
28. Catz SD, Johnson JL: Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. *Oncogene*, 2001; 20: 7342-7351.
29. Tagawa, H.; Karnan, S.; Suzuki, R.; Matsuo, K.; Zhang, X.; Ota, A.; Morishima, Y.; Nakamura, S.; Seto, M. Genome-wide array-based CGH for mantle cell lymphoma: Identification of homozygous

deletions of the proapoptotic gene BIM. *Oncogene*, 2005; *24*: 1348–1358.
30. Cimmino, A.; Calin, G.A.; Fabbri, M.; Iorio, M.V.; Ferracin, M.; Shimizu, M.; Wojcik, S.E.; Aqeilan, R.I.; Zupo, S.; Dono, M.; et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc. Natl. Acad. Sci. USA*, 2005; *102*: 13944–13949.
31. Agarwal, B; Naresh, K. Bcl-2 Family of Proteins in Indolent B-Cell Non-Hodgkin's Lymphoma: Study of 116 Cases. *American Journal of Hematology*, 2002; 70: 278–282.
32. Leich, E.; Salaverria, I.; Bea, S.; Zettl, A.; Wright, G.; Moreno, V.; Gascoyne, R.D.; Chan, W.C.; Braziel, R.M.;Rimsza, L.M.; et al. Follicular lymphomas with and without translocation t(14;18) differ in gene expression profiles and genetic alterations. *Blood*, 2009; *114*: 826–834.
33. Takeshita, M; Kikuchi, M; Huang, GS; Li, Q; Ohshima, K. Bcl2-negative follicular lymphomas frequently have Bcl6 translocation and/or Bcl6 or p53 expression. *Pathol Int*, 2007; 57(3): 148-52.
34. Wang, X; Medeiros, L; Bueso-Ramos, C; Tang, G; Wang, S; Oki, Y; Desai, P; Khoury, J; Miranda, R; Tang, Z; Reddy, N; Li, S. P53 expression correlates with poorer survival and augments the negative prognostic effect of MYC earrangement, expression or concurrent MYC/BCL2 expression in diffuse large B-cell lymphoma. *Modern Pathology*, 2017; 30: 194–203.
35. Xie Y, Bulbul MA, Ji L, et al. p53 expression is a strong marker of inferior survival in de novo diffuse large B-cell lymphoma and may have enhanced negative effect with MYC coexpression: a single institutional clinicopathologic study. *Am J Clin Pathol*, 2014; 141: 593–604.
36. Bilalovic, N; Blystad, A; Golouh, R; Nesland, J; Selak, I; Trinh, D; Torlakovic, E. Expression of bcl-6 and CD10 Protein Is Associated With Longer Overall Survival and Time to Treatment Failure in Follicular Lymphoma. *Am J Clin Pathol*, 2004; 121: 34-42.
37. Hans CP, Weisenburger DD, Greiner TC, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood*, 2004; 103: 275-282.
38. Colomo L, Lopez-Guillermo A, Perales M, et al. Clinical impact of the differentiation profile assessed by immunophenotyping in patients with diffuse large B-cell lymphoma. *Blood*, 2003; 101: 78-84.
39. Lossos IS, Jones CD, Warnke R, et al. Expression of a single gene, *BCL-6*, strongly predictssurvival in patients with diffuse large B-cell lymphoma. *Blood*, 2001; 98: 945-951.
40. Holler, S; Horn, H; Lohr, A; Mader, U; Katzenberger, T; Kalla, J; Bernd, HW; Went, P; Ott, M; Muller-Hermenlink, HK; Rosenwald, A; Ott, G. A cytomorphological and immunohistochemical profile of aggressive B-cell lymphoma: high clinical impact of a cumulative immunohistochemical outcome predictor score. *J Hematopathol*, 2009; 2: 187–194.
41. Cattoretti G, Pasqualucci L, Ballon G, et al. Deregulated BCL6 expression recapitulates the pathogenesis of human diffuse large B cell lymphomas in mice. *Cancer Cell*, 2005; 7: 445-455.
42. Saito, M., Novak, U., Piovan, E., Basso, K., Sumazin, P., Schneider, C., et al. BCL6 suppression of BCL2 via Miz1 and its disruption in diffuse large B cell lymphoma. *Proc. Natl. Acad. Sci. U.S.A.*, 2009; 106: 11294–11299.
43. Kienle D, Kröber A, Katzenberger T, et al. VH mutation status and VDJ rearrangement structure in mantle cell lymphoma: *correlation* with genomic aberrations, clinical characteristics, and outcome. *Blood*, *2003*; 102: 3003–3009.
44. Orchard J, Garand R, Davis Z, et al. A subset of t(11;14) lymphoma with mantle cell features displays mutated IgVH genes and includes patients with good prognosis, non nodal disease. *Blood*, 2003; 101: 4975–4981.