Original Article doi: 10.5146/tjpath.2018.01430

Evaluation of the Prognostic Importance of c-Myc and Bcl-2 Expressions and the Presence of Epstein-Barr Virus in Classical Hodgkin Lymphoma

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ABSTRACT

Objective: Although classical Hodgkin lymphoma (cHL) has a relatively good prognosis, it also entails different treatment responses and involves patients who have different clinical courses. Our aim was to investigate c-Myc, Bcl-2 and EBV biomarkers in cHL and their relationship with the IPS score.

Material and Method: c-Myc and Bcl-2 immunohistochemical staining with EBER in situ hybridization (EBER-ISH) was applied to the paraffin sections of 94 cases diagnosed as cHL. These cases were classified into two groups as low and high clinical symptoms according to the International Prognostic Scores (IPS).

Results: Positive results were obtained in 83 (88.3%) cases with c-Myc and 39 (43.5%) cases with Bcl-2 while EBER-ISH was found positive in 42 (44.7%) cases. No difference was found between the groups of low/high IP scores with respect to the positive or negative results of EBER-ISH, Bcl-2 and c-Myc. When Bcl-2 and c-Myc positive cases were grouped together and compared to the IP scores of the remaining cHL cases, again no difference was seen. Extranodal involvement and bone marrow involvement was observed in 25 (26.5%) and 9 (9.5%) cases, respectively. Similarly, no statistically significant differences was found between these groups according to their positivity with EBER-ISH, Bcl-2 and c-Myc.

Conclusion: We could not find any relationship between Bcl-2, c-Myc and EBER-ISH positivity and the low/high IPS groups in cHL. New studies with larger series are needed in which more precise cut-off values are used and clinically and biologically heterogeneous groups of cHL patients are determined more clearly.

Key Words: Hodgkin lymphoma, c-Myc, Bcl-2, EBER

INTRODUCTION

Hodgkin lymphoma (HL) is a partially homogeneous neoplastic disorder due to its relatively indolent clinical course (1). However its histomorphological findings, immune phenotype, genotype, relationship with viruses and its responses to treatment may vary (2-7). Several clinical parameters (age, stage, subtype, tumor burden, etc.) are used as predictive factors in the HL patients' lifespan and responses to treatment (8-10). Until now, genetic and molecular steps in the progression of HL have not been completely understood. Genetic and immunohistochemical studies have been conducted into multiple cell cycle regulators in the pathogenesis of HL. These studies include numerous biological markers such as apoptosis inhibitors (Bcl-2, bcl-X), tumor suppressors (p-53, RB, etc.), proto-oncogenes (c-Myc, NOTCH1, IRF4, etc.), as well as the Epstein-Barr virus (11-18).

Certain biological markers are known to be predictive factors for success of treatment and recovery chance of some non-Hodgkin lymphoma (NHL) types. It has been shown that the presence of Bcl-2, c-Myc, and Bcl-6 expressions in diffuse large B-cell lymphomas are related with poor prognosis (19,20) and the presence of p-53 mutation in mantle cell lymphomas is a predictor of aggressive behaviour (21,22). It has also been determined that presence of EBV in T-cell NHLs is related with poor prognosis (23,24).

Several prognostic models are used to classify lymphoma patients into groups of high and low risk. The International Prognostic Score (IPS) consists of a combination of various

(Turk Patoloji Derg 2018, 34:199-206)

Received : 12.03.2018 **Accepted :** 05.04.2018

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clinical and laboratory parameters and the risk of a patient is determined by these parameters (25,26). In fact, even in risk groups as determined by IPS, different treatment results may be obtained. Some researchers have tried to develop research models that use biological markers together with clinical prognostic models. Nevertheless, the independent prediction accuracy of these indicators is limited and not widely accepted.

In this study, we aimed to put forward c-Myc, Bcl-2 and EBV status of cHL and whether these indicators are related with prognostic findings as determined by IPS.

MATERIAL and METHODS

Patients: Ninety-four cases diagnosed as classical Hodgkin Lymphoma (cHL) between 2007 and 2016 were included in the study. The cases were re-evaluated by two pathologists (A.K. and V.Ö.) according to the 2017 WHO classification criteria. Diagnostic blocks with cHL were chosen for immunohistochemical staining and EBER (Epstein-Barr virus-encoded RNA) in situ hybridization tests. Clinical parameters of prognostic importance were obtained through the computer files and data in the hematology and oncology clinics where these patients were monitored.

Clinicopathological data (age, sex, histological subtype, clinical stage, bone marrow involvement, prognostic score, the presence and date of relapse, together with the presence of extranodal involvement) of the cases were obtained from the patient files and were categorised by using IPS (International Prognostic Score). IPS scores were coded as 0, 1, and 2 representing "low" and 3 and above representing "high" risk.

Immunohistochemistry

3-4 μm thick sections were placed on slides covered with poly-L-lysine out of the blocks of cHL-diagnosed formalin-fixed paraffin-embedded biopsy samples for immunohistochemical studies. The standard protocol was applied by the Benchmark GX IHK/ISH (Ventana) automatic staining device for the anticors of c-Myc (clone Y69, Ventana) and Bcl-2 (clone 2/100/05, Novocastra). For both of the markers, stainings below 40% were regarded as "negative" and those above 40% were regarded as "positive" in Hodgkin/Reed-Sternberg (HRS) cells.

EBER (EBV-encoded RNA) in Situ Hybridization

EBER-ISH was applied to all cases using ISH iVIEW Blue Detection Kit (Ventana) with silver labeled oligonucleotide probes (INFORM EBER Probe, Ventana) in the Benchmark GX IHK/ISH (Ventana) autostainer device. After deparaffinization and soft and standard cell healing, 12 minutes of protease 3, and the INFORM EBER steps were applied; then 6 minutes of red counterstain was performed on the background and dark-blue nuclear staining was considered to be positive in HRS cells.

Statistical Analysis

The SPSS for MacOS program was used to analyse the data. Discrete numeric variables of descriptive statistics were shown as mean±standard deviation or median (minimum-maximum), nominal variables were shown as the number of cases and percentage (%).

Nominal variables were evaluated by using Pearson's Ki-square or Fisher's exact test. The results for $p<0.05$ were regarded as statistically significant.

RESULTS

Samples taken from a total of 94 patients of whom 33 were female (35.1%) and 61 were male (64.9%) and whose median age was 40.5 years (ranging between 15 and 75 years). Of the cases, 55 (58.5%) were nodular sclerosing, 19 (20.2%) were mixed cellular, 15 (16.0%) were lymphocyte rich and 5 (5.3%) of them were lymphocyte poor subtype.

Examined IPS scores were ranged between 0 and 7, and the median IPS score was 2.0 (IQR=2.0). 63 (67.0%) of the cHL cases were classified as low and 31 (32.9%) as high risk categories.

While the staining rate for c-Myc ranged between 0% and 100%, the staining median for c-Myc was calculated as 70.0% (IQR=20.0) (Figure 1A-B). The Bcl-2 staining ranged between 10% and 100%; and staining median was 0% (IQR=60%) (Figure 2).

Values below 40% were regarded as "negative" while 40% and above were regarded as positive for both of the markers. Accordingly, 83 (88.3%) cases with c-Myc were seen to be positive while 39 (41.5%) cases with Bcl-2 were evaluated as positive. EBV was determined as positive in 42 (44.7) cases (Figure 3).

No statistical significant difference was found, concerning EBV, Bcl-2 and c-Myc positivity between the low and high risk groups of IPS ($p>0.05$) (Table I).

Extranodal involvement was found in 25 (26.5%) of the cases. No difference was found about Bcl-2 and c-Myc staining rates with relation to extranodal involvement ($p>0.05$) (Table II). However, 21 (84%) of 25 cases with extranodal involvement also had positive staining with c-Myc. The rate of extranodal involvement in EBV positive cases was significantly higher than in EBV negative cases ($\chi 2 = 5.413$; $p=0.023$). When all other factors (such as age,

Table I: The staining rates with c-Myc, Bcl-2 and EBER-ISH in IP score groups

		IP Score Group				
Marker	**Result**	**Low**	**High**	**Total**	χ^2	**p**
c-Myc	Negative	8 (12.7)	3 (9,7)	**11 (11.7)**	0,184	0.668
	Positive	55 (87.3)	28 (90,3)	**83 (88.3)**		
	Total	**63 (100.0)**	**31 (100.0)**	**94 (100.0)**		
Bcl-2	Negative	38 (60.3)	17 (54.8)	**55 (58.5)**	0.257	0.612
	Positive	25 (39.7)	14 (45.2)	**39 (41.5)**		
	Total	**63 (100.0)**	**31 (100.0)**	**94 (100.0)**		
EBER-ISH	Negative	37 (58.7)	15 (48.4)	**52 (55.3)**	0.899	0.343
	Positive	26 (41.3)	16 (51.6)	**42 (44.7)**		
	Total	**63 (100.0)**	**31 (100.0)**	**94 (100.0)**		

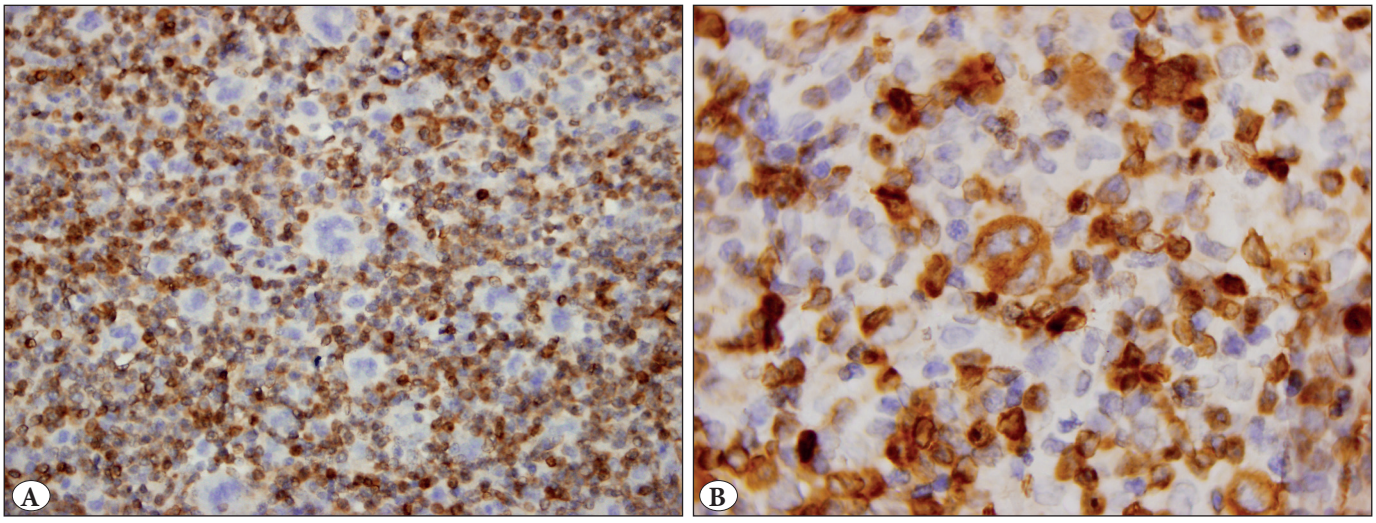

Figure 1: A) In Bcl-2 immunohistochemical staining, lymphocytes around HRS cells shows positive staining in the form of rosettes (IHC; x40). **B)** Cytoplasmic positive staining in HRS cells with Bcl-2 (IHC; x100).

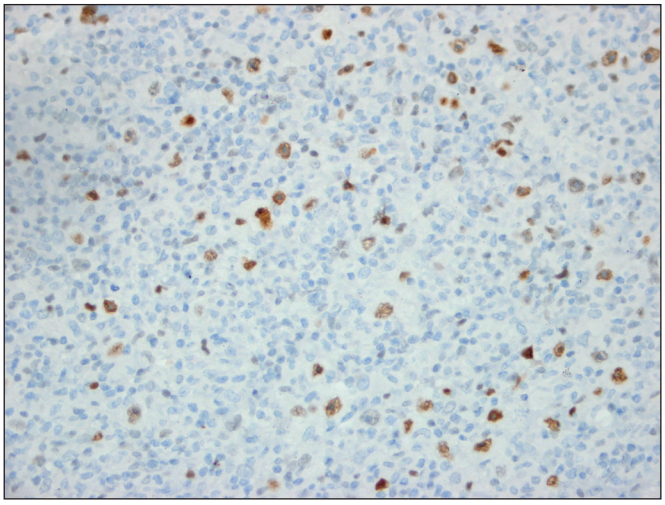

Figure 2: In c-Myc immunohistochemical staining, common positive staining is observed in HRS cells' nucleus (IHC; x400).

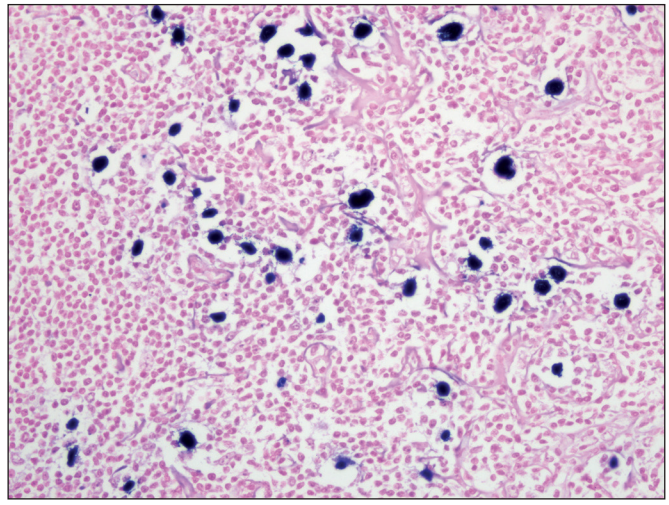

Figure 3: In EBER ISH, dark-blue nuclear staining was considered to be positive in HRS cells (CISH; x400).

gender and the stage of illness) are assumed stable, the risk of extranodal involvement in EBV positive cases is OR=2.98 (95% CI: 1.16 - 7.65) times higher than in EBV negative cases.

Bone marrow involvement was seen in 9 (9.5%) of the cases. No difference was found concerning the staining rates of c-Myc, Bcl-2 and EBER-ISH in relation to bone marrow involvement (p>0.05) (Table III). Positive stainig with c-Myc was found in 7 of the 9 cases with bone marrow involvement, and positive Bcl-2 was found in 3 of them.

EBV was found to be positive in 42 (44.6%), and negative 52 (55.3%) of the cases with EBER-ISH. No significant relationship was detected between EBER-ISH, c-Myc and Bcl-2 expressions (Table IV).

To observe any difference according the IP scores between Bcl-2+/c-Myc+ patients and the remaining patients, cases

Table II: The staining rates of c-Myc, Bcl-2 and EBER-ISH in relation to extranodal involvement

		Extranodal Involvement				
Marker	**Result**	**No**	**Yes**	**Total**	**χ^2**	**P**
c-Myc	Negative	7 (10.1)	4 (16.0)	**11 (11.7)**	-	0.475
	Positive	62 (89.9)	21 (84.0)	**83 (88.3)**		
	Total	**69 (100.0)**	**25 (100.0)**	**94 (100.0)**		
Bcl-2	Negative	42 (60.9)	13 (52.0)	**55 (58.5)**	0.595	0.441
	Positive	27 (39.1)	12 (48.0)	**39 (41.5)**		
	Total	**69 (100.0)**	**25 (100.0)**	**94 (100.0)**		
EBER-ISH	Negative	43 (62.3)	9 (36.0)	**52 (55.3)**	5.143	0.023
	Positive	26 (37.7)	16 (64.0)	**42 (44.7)**		
	Total	**69 (100.0)**	**25 (100.0)**	**94 (100.0)**		

Table III: The staining rates of c-Myc, Bcl-2 and EBER-ISH in relation to bone marrow involvement

		Bone Marrow Involvement				
Marker	**Result**	**No**	**Yes**	**Total**	**χ^2**	**p**
c-Myc	Negative	9 (10,6)	2 (22.2)	**11 (11.7)**	-	0.283
	Positive	76 (89.4)	7 (77.8)	**83 (88.3)**		
	Total	**85 (100.0)**	**9 (100.0)**	**94 (100.0)**		
Bcl-2	Negative	49 (57.6)	6 (66.7)	**55 (58.5)**	-	0.731
	Positive	36 (42.4)	3 (33.3)	**39 (41.5)**		
	Total	**85 (100.0)**	**9 (100.0)**	**94 (10.0)**		
EBER-ISH	Negative	49 (57.6)	3 (33.3)	**52 (55.3)**	-	0.290
	Positive	36 (42.4)	6 (66.7)	**42 (44.7)**		
	Total	**85 (100.0)**	**9 (100.0)**	**94 (100.0)**		

Table IV: The staining rates of c-Myc and Bcl-2 in relation to the EBER-ISH positivity

Marker	**Result**	**EBER-ISH Negative**	**EBER-ISH Positive**	**Total**	**χ^2**	**p**
c-Myc	Negative	7 (13.5)	4 (9.5)	11 (11.7)	-	0.749
	Positive	45 (86.5)	38 (90.5)	83 (88.3)		
	Total	**52 (100.0)**	**42 (100.0)**	**94 (100.0)**		
Bcl-2	Negative	27 (51.9)	28 (66.7)	55 (58.5)	2.080	0.149
	Positive	25 (48.1)	14 (33.3)	39 (41.5)		
	Total	**52 (100.0)**	**42 (100.0)**	**94 (100.0)**		

Table V: The staining rates for IPS groups of Bcl-2 +/c-Myc + and the others

		IPS Score Group				
Marker	**Result**	**Low**	**High**	**Total**	χ^2	**p**
Others	Negative	40 (63.5)	18 (58.1)	**58 (61,7)**	0.259	0.611
c-Myc(+) +	Positive	23 (36.5)	13 (41.9)	**36 (38.3)**		
Bcl-2(+)	**Total**	**63 (100.0)**	**31 (100.0)**	**94 100.0)**		

were reclassified into two groups: Bcl-2+/c-Myc+ cases and other cases (Bcl-2+/c-Myc-; Bcl-2-/c-Myc-; and Bcl-2-/c-Myc+) (Table V). There was no difference between the low and high IPS groups (p>0.05).

DISCUSSION

A considerable number of HL cases are tumors that respond to first-line therapy (27). However,, in approximately 20% to 30%, relapse, treatment complications and death from the disease occur (28, 29). Additionally, a sufficient response to standard treatment could not be obtained in more than one third of HL cases. The factors affecting treatment response in this patient group may include advanced stage disease, the presence of B symptoms, oncogenic proteins, or molecular abnormalities in the suppressor protein panels. In several studies, a comparison has been made between the biological markers that have potential routine use and the clinical results and the effects on known prognostic factors have been researched (3,30,31).

Several proto-oncogenes may affect lymphoid malignancies and one of the most important of these is c-Myc (32). c-Myc plays role in various cellular functions such as cell cycle, cell growth, cell metabolism, biosynthesis, adhesion and also the control of mitochondrial function. The determination of c-Myc protein expression and gene translocation is important in the diagnosis of lymphomas and specifying clinical sequence of aggressive B cell lymphomas (33,34). It has been demonstrated that c-Myc translocation is related with a poor prognosis in the subgroups of diffuse large B cell lymphoma patients (20,35). Additionally, it has also been shown that diffuse large B-cell lymphoma patients with co-expression of c-Myc, Bcl-6 and Bcl-2 proteins have a poor prognosis and do not respond to routine chemotherapy (19,20,35). The presence of c-Myc has been searched in a limited number of studies concerning HL and positive staining was shown for the major part of those cases (36,37). We aimed to show c-Myc expression status in cHL and to search whether there is any risk in terms of IPS risk groups. However, we were unable to obtain any significant difference between the low and high risk IPS groups. Besides, in 61 (68.5%) of 89 cases, IPS was found to be low. Moreover, there was no bone marrow involvement in 82 (92.1%) and no extranodal involvement in 68 (76.4%) out of these 89 cases.

Biological markers such as Bcl-2, BAX, Bcl-X and p53 are proteins that organize apoptosis. High staining rates of Bcl-2 in HRS cells have been associated with negative consequences in HL patients in some studies (5, 12, 38). In our study, we did not find any significant relationship between Bcl-2 expression in HRS and the low and high risk IPS groups. Furthermore, 25 (64,1%) out of 39 Bcl-2 positive cases were grouped as low risk IPS. Also, out of 39 cases that were stained Bcl-2 positive, 27 (69.2%) and 36 (92.3%) cases had no extranodal involvement and no bone marrow involvement, respectively. The chosen method and the cut-off value regarding positivity may have resulted differently in previous studies. There aren't many articles concerning the staining threshold of c-Myc and Bcl-2 expressions in HL. In certain studies mentioning significant differences, 10% has been used as a threshold for Bcl-2 positivity, regarding the clinical consequences of HL (3,5,38). In studies that assumed the threshold as 20% and above, no relationship has been found in the same context (12,39). However, diffuse large B-cell lymphoma studies concerning c-Myc and Bcl-2 expression, usually 40% and above positivity is used as cut-off point (40,41). Hence, we preferred to use the same scope for our study, but this may be the cause that why we could not find any significant relationship between Bcl-2/c-Myc expressions and IP scores. This leads to stating the limitation of our study, not including either applied therapy or the results of the treatment for the patients .

In the literature, the Epstein-Barr virus (EBV) has been determined to be positive in 47.9% of cHL patients (42). Whether there is a relationship between EBV infection and the advanced clinical stages of cHL is highly debatable. While some studies (43-45) have found an important correlation between EBV and the advanced stage of cHL, other studies have not found such a relationship (46-48). We found that 42 (44.7%) of cHL cases were EBV positive. We could not find any difference between positive and negative EBV cases in terms of IPS and bone marrow involvement, but, there was significantly high extranodal

involvement in those cases which were EBV positive. Besides, no relationship was found between EBER-ISH and Bcl-2/c-Myc expressions in our series. These markers have been defined as independent factors from each other in other literature (6,15,49).

Finally, we also researched whether co-expression of c-Myc and Bcl-2 is related with low and high risk IP scores, but we could not find any significant result.

It is concluded that future studies with larger series may lead to finding an optimal cut-off value for c-Myc and Bcl-2 and can provide more prognostic information in cHL, including determining treatment-resistant/high-risk cases.

CONFLICT of INTEREST

The authors declare no conflict of interest.

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