ORIGINAL ARTICLE

The Prognostic Importance of Immunohistochemical Biomarkers in Diffuse Large B-Cell Lymphoma

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ABSTRACT

Objective: *Molecular methods have practical difficulties in identifying sub-groups of diffuse large B-cell lymphoma (DLBCL) in routine clinical practice. The goal of this study was to sub-classify DLBCL patients into sub-groups by immunohistochemical method and to evaluate the effects of sub-groups on prognosis.*

Methods: *For this purpose, the lymph node biopsy specimens of 40 patients with DLBCL have stained with monoclonal antibody immunostains of* cluster of differentiation 10*, B-cell lymphoma 6 and multiple myeloma oncogene 1 (MUM1).*

Results: *As a result, 6 (15%) patients have germinal centre B-cell like (GCB) phenotype and 34 (85%) patients have non-GCB phenotype. The overall survival (OS) and event-free survival (EFS) was 31.00 ± 15.49 months and 27.66 ± 17.95 months in GCB phenotype, respectively. The OS and EFS were 23.79 ± 17.82 months and 20.97 ± 17.12 months in non-GCB phenotype, respectively.*

Conclusion: *Multiple myeloma oncogene 1 has reached statistical significance among immunostains, and was found negatively correlated with OS and EFS. If these markers are standardized in the future, more accurate treatment schedules will be determined.*

Keywords: Diffuse large B-cell lymphoma, immunohistochemistry methods, prognosis

INTRODUCTION

Diffuse large B-cell lymphomas (DLBCL) accounts for 30%–40% of adult non-Hodgkin's lymphomas. Diffuse large B-cell lymphomas is heterogeneous both clinically and morphologically. A proportion of DLBCL patients is cured with conventional therapies such as rituximab plus CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone); however, the majority of patients experience a poor outcome of treatment. Because, the International Prognostic Index (IPI) alone is not sufficiently powerful to separate patients who will be cured by conventional therapy from those who have refractory or relapsing disease, so it is important to identify high-risk patients that may benefit from novel therapeutic approaches or more aggressive therapies at diagnosis.

The first gene expression profiling (GEP) studies have identified different sub-groups with chemotherapy responses and total life span, which are germinal centre B-cell like (GCB), activated B-cell like (ABC) and unclassified DLBCL (1, 2). It was shown that patients with GCB have a better survival than ABC independent of the IPI (1).

However, molecular methods are too expensive and have practical difficulties in routine clinical practice. Thus, several studies investigated immunohistochemical (IHC) methods to sub-classify DLBCL into molecularly distinct and prognostically significant sub-groups (3, 4). Since the results of these studies are conflicting and used monoclonal antibodies are still not standardized, so new approaches are needed in this field.

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The goal of this study was to sub-classify DLBCL patients into sub-groups based on the Hans classification and to evaluate the effect of sub-groups on prognosis independent of the IPI. For this purpose, the lymph node biopsy specimens of 40 patients have stained with monoclonal antibody immunostains of CD10 (cluster of differentiation 10), Bcl-6 (B-cell lymphoma 6) and MUM1/IRF4 (multiple myeloma oncogene 1/Interferon Regulating Factor 4).

MATERIALS AND METHODS

The study was conducted in a prospective manner in Cumhuriyet University Medical School between December 2011 and January 2013. The departments of haematology and pathology have contributed to this study. All patients have provided their written consent for the participation in this study. The study was approved by the local Ethics Committees and was in accordance with the Declaration of Helsinki.

A total of 40 patients were enrolled in the study. The participants received an anthracycline-containing chemotherapy and/or rituximab regimen. The diagnostic lymph node biopsy specimens of 40 patients with DLBCL were found by the archive of pathology. For the tissue microarray (TMA), haematoxylin and eosin-stained sections from each paraffin-embedded, formalin-fixed block were used to define diagnostic areas. The sections of formalin-fixed paraffin-embedded biopsies were deparaffinised in xylene. Following deparaffinization of the sections, ethyl alcohol was used for hydration. Then sections were washed in distilled water and boiled in microwave oven in 0.01 M EDTA buffer (pH = 8.0) for Bcl-6 and in 0.01 M citrate buffer (pH = 6.0) for CD10 and MUM1. Then sections were washed again with distilled water.

The specimens were treated to phosphate buffer saline (PBS) twice for 3 minutes. Then they were treated to hydrogen peroxide (3%) for 15 minutes and again PBS twice for 3 minutes. Protein blockade was performed for 10 minutes. After washing, the sections were stained with antibodies to CD10 [CALLA (Neutral Endopeptidase) Ab-2 (Clone 56C6), Mouse Monoclonal Antibody, Ready-to-Use, Thermo Scientific, USA], Bcl-6 [BCL6 (Clone LN22), Mouse Monoclonal Antibody, Ready-to-Use, Thermo Scientific, USA], and MUM1 [MUM1 Protein (Clone MRQ-43), Rabbit Monoclonal Antibody, Working dilution: 1:50, Zeta Corporation, USA]. They were again treated to PBS twice for 3 minutes and then streptavidin peroxidase was performed by 15 minutes. Then the sections were incubated with aminoethyl carbazole chromogen for 15 minutes. The colouring specimens were washed in distilled water and waited for a minute in haematoxylin. Finally, the specimens were covered with a covering device.

Each specimen was evaluated independently by two pathologists for the percentage of tumour cells staining by polarized light microscopy. For each case, the section with the highest percentage of tumour cells stained was used for analysis. Cases were considered positive if 30% or more of the tumour cells were stained with an antibody. Immunostain results for CD10, Bcl-6 and MUM1 were used to sub-classify the cases. The cases were classified into two groups: GCB or non-GCB. Figs. 1 and 2 are demonstrative examples for each group. Cases were assigned to the GCB group if CD10 alone was positive or if both Bcl-6 and CD10 were positive. If both Bcl-6 and CD10 were negative, the case was assigned to the non-GCB sub-group. If Bcl-6 was positive and CD10 was negative, the expression of MUM1 determined the group. If MUM1 was negative, the case was assigned to the GCB group, and if MUM1 was positive, the case was assigned to the non-GCB group.

Overall survival (OS) time was calculated from the date of diagnosis to the date of death or last follow-up. Event-free survival (EFS) was calculated from the time of diagnosis to the date of progression, death due to any cause or last visit.

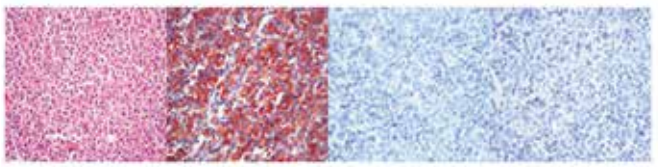

Fig. 1: A–D. GCB phenotype (original magnification, ×40). H&E section of diffuse large B-cell lymphoma showing sheets of large nucleolated lymphoid cells (A). Positive staining of CD10 (B). Negative for Bcl-6 (C). Negative for MUM1 (D).

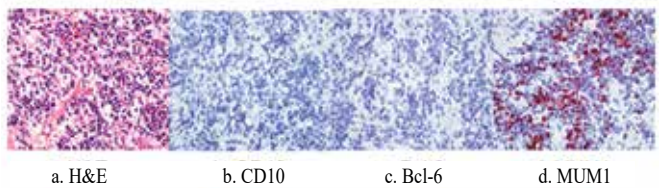

Fig. 2: A–D. Non-GCB phenotype (original magnification, ×40). H&E section of diffuse large B-cell lymphoma showing sheets of large nucleolated lymphoid cells (A). Negative for CD10 (B). Negative for Bcl-6 (C). Positive staining of MUM1 (D).

Statistical analysis

All statistical analyses were performed using SPSS version 14.0 (SPSS, Chicago, IL, USA). Descriptive statistics were calculated for each of the variables. Data were expressed as mean ± standard deviation (normally distributed data), median and interquartile range (non-normally distributed data) or as percentage frequencies. For survival analysis, univariate Kaplan–Meier analysis were performed. Differences among variables were evaluated by Mann–Whitney U and Pearson Chi-square tests. *p* values of less than 0.05 were regarded as significant.

RESULTS

A total of 40 cases [27 (67.5%) male, 13 (32.5%) female] were evaluated. The median age of the patients was 61 years (range; 23–85). The laboratory and clinical characteristics of the patients are exhibited in Table 1. According to the stage of the disease, of the patients, 7 (17.5%) were in stage 1, 8 (20.0%) were in stage 2, 19 (47.5%) were in stage 3, and 6 (15.0%) were in stage 4. Namely, the majority of patients had referred at late stages of the disease.

Table 1: The laboratory and clinical characteristics of the patients

Characteristics	Mean ± SD
Hb (g/dL)	13.06 ± 2.30
WBC ($\times 10^9$/L)	8.59 ± 3.64
PLT ($\times 10^9$/L)	285.03 ± 117.57
LDH (IU/L)	257.95 ± 123.47
Ki-67 proliferation index (%)	57.00 ± 16.20
BMI (kg/m^2)	26.28 ± 6.61
B symptoms, n (%)	21 (52.5%)
Liver involvement, n (%)	6 (15.0%)
Spleen involvement, n (%)	11 (27.5%)
Bone marrow involvement, n (%)	8 (20.0%)
Nodal involvement, n (%)	25 (62.5%)
Extranodal involvement, n (%)	15 (37.5%)

BMI = body mass index; Hb = haemoglobin; LDH = lactate dehydrogenase; PLT = platelets; WBC: white blood cell count

Monoclonal antibody immunostains of CD10 was positive in 5 (12.5%) patients, Bcl-6 was positive in 2 (5.0%) patients and MUM1 was positive in 15 (37.5%) patients. Bcl-2 was stained in 18 patients, and of these patients, 12 (66.7%) were positive. According to these immunostains results, 6 (15.0%) patients were classified as GCB phenotype and 34 (85.0%) patients were classified as non-GCB phenotype.

Since therapeutic outcomes were analysed, complete remission, partial remission, stable disease and progressive disease were obtained in 24 (60.0%), 4 (10.0%), 10 (25.0%), and 2 (5.0%) patients, respectively. Thirty-one (77.5%) of the patients were treated with rituximab plus chemotherapy. Of the patients, 6 (15.0%) relapsed and they were treated with salvage regimens. Thirty-one (77.5%) of the patients are still alive and being followed-up regularly. Of the patients, 9 (22.5%) died of unrelated causes, and all deaths were in non-GCB group.

The median OS and EFS rates were 25 months (range; 1–76) (Fig. 3) and 22 months (range; 0.8–68), respectively. Although the OS and EFS rates were higher in GCB group than non-GCB group, but the differences between groups did not reach statistical difference. However, the life span was longer in GCB phenotype.

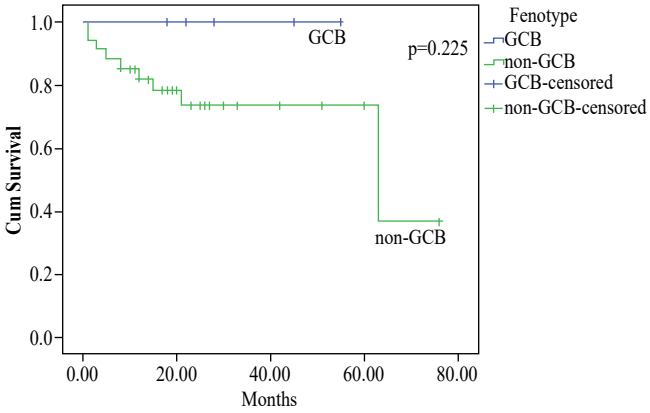

Fig. 3: Overall survival curves comparing GCB phenotype with non-GCB phenotype.

International Prognostic Index was found '0' in 2 (5.0%) patients, '1' in 14 (35.0) patients, '2' in 8 (20.0%) patients, '3' in 11 (27.5%) patients and '4' in 4 (10.0%) patients. In our study, the IPI score predicted OS ($p = 0.004$) and EFS ($p = 0.001$) when comparing those with low (0–2) *versus* high (3–5) scores. When we consider ECOG performance status, ECOG was '0' in 12 (30.0%) patients, '1' in 17 (42.5%) patients, '2' in 8 (20.0%) patients and '3' in 3 (7.5%) patients. The mean Ki-67 proliferation index was found 51.16 ± 17.15 and 57.50 ± 16.24, in GCB and non-GCB groups, respectively. However, there was not a statistical difference between groups ($p = 0.606$). A detailed summary of IHC stain results, IPI, phenotypes and their effects on survival rates were displayed in Table 2. Only MUM1 has reached statistical significance among immunostains. It was negative correlated with OS (Fig. 4) and EFS (Fig. 5).

Table 2: The association between survival rates and immunohistochemical stain results, IPI and phenotypes

	n (%)	OS (mean ± SD)	*p* value	EFS (mean ± SD)	*p* value
CD10					
Positive	5 (12.5)	33.60 ± 15.78	0.135	29.60 ± 19.35	0.336
Negative	35 (87.5)	23.62 ± 17.58		20.88 ± 16.87	
Bcl-6					
Positive	2 (5.0)	23.00 ± 31.11	NA	22.50 ± 31.81	NA
Negative	38 (95.0)	24.97 ± 17.21		21.94 ± 16.85	
MUM1					
Positive	15 (37.5)	16.46 ± 12.84	*0.009*	14.13 ± 13.59	*0.015*[a]
Negative	25 (62.5)	29.92 ± 18.18		26.68 ± 17.62	
Bcl-2					
Positive	12 (66.7)	19.00 ± 11.04	0.133	17.00 ± 12.35	0.075
Negative	6 (33.3)	37.83 ± 23.21		34.16 ± 21.06	
IPI					
0, 1, 2	23 (57.5)	31.91 ± 18.57	*0.004*	30.17 ± 17.59	*0.001*
3, 4, 5	17 (42.5)	15.35 ± 10.23		10.88 ± 8.19	
Phenotype					
GCB	6 (15.0)	31.00 ± 15.49	0.225	27.66 ± 17.95	0.370
non-GCB	34 (85.0)	23.79 ± 17.82		20.97 ± 17.12	

EFS = event-free survival; IPI = International Prognostic Index; NA = not applicable; OS = overall survival.
[a]Italic values mean *p* values are less than 0.05.

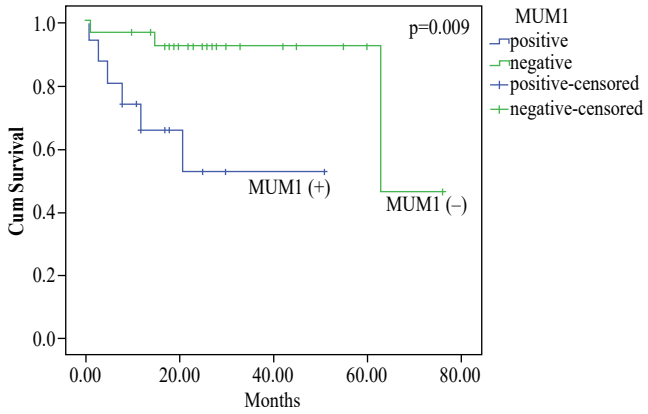

Fig. 4: Overall survival of MUM1 positive *versus* MUM1 negative cases.

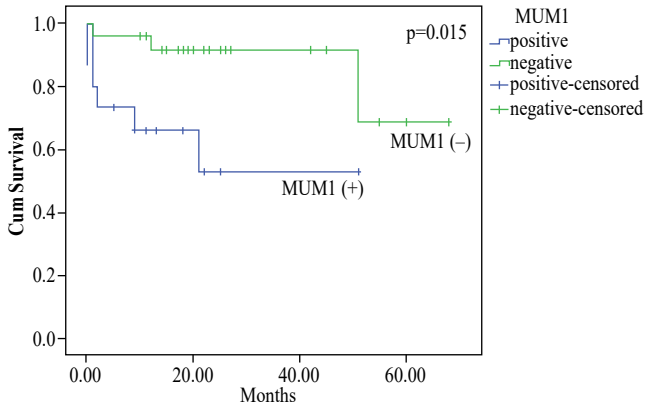

Fig. 5: Event free survival of MUM1 positive *versus* MUM1 negative cases.

DISCUSSION

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease both clinically and morphologically. Using GEP studies, DLBCL were divided into prognostically significant sub-groups termed GCB and ABC (1). Later, a third group called type 3, was determined in gene expression studies (5). Although a few genes can be used to identify sub-groups by gene expression technology, however, practical difficulties in routine clinical use, the requirement of fresh or frozen tissue with an adequate amount of RNA and difficulties in obtaining tissue for routine histology restrict its use (1, 5). In contrast, TMA is a cost-effective method and allows the rapid evaluation of IHC staining of multiple tumours in a single tissue section. Evaluation of the staining results is also easier because each tissue section can be completely viewed under a light microscope, and each case can be finalized in a short time. Furthermore, TMA allows preservation of the tissue in paraffin blocks for future studies. Therefore, IHC offers practical utilities in identifying sub-groups of DLBCL.

In IHC studies, B-cell differentiation antigens have been considered. Of the B-cell differentiation antigens, CD10 and Bcl-6 are expressed in the germinal centre cells (6). In contrast, MUM1 is expressed in plasma cells and a minor subset of germinal centre cells (10–12). Hans *et al* (3) have used monoclonal antibody immunostains of CD10, Bcl-6 and MUM1 to sub-classify DLBCL patients. They found that the GCB and non-GCB subtypes of DLBCL can be accurately predicted using these three immunostains. Since they compared IHC results with the cDNA microarray, the IHC panel reproduced the gene expression results in 71% of GCB and 88% of non-GCB cases and predicted for survival in a similar manner. Their study was the first to correlate sub-classification by gene expression with sub-classification by protein expression in DLBCL. Based on the Hans classification, 6 (15.0%) patients were classified as GCB phenotype, and 34 (85.0%) patients were classified as non-GCB phenotype in our study.

CD10 is a membrane-associated, neutral endopeptidase that is expressed in a variety of human tissues (6). Previous studies have suggested that CD10 expression in DLBCL may be a predictor of inferior survival (7, 8). Some studies using IHC methods have found that CD10 expression is associated with significantly improved OS (9, 10). Whereas other studies have found no difference in outcomes of patients in terms of CD10 expression (11, 12). In our study, we could not demonstrate a difference between CD10 expression and survival.

Bcl-6 is a protein that acts as a transcriptional repressor, and is expressed in germinal centre B cells (13). Immunohistochemical studies of Bcl-6 expression and its relationship to outcome in DLBCL are limited in number. Several studies reported no difference in OS related to Bcl-6 expression (14, 15). Although, the others found Bcl-6 expression to be associated with a better OS (16, 17). We could not perform a statistical evaluation because of the very limited Bcl-6 positive number of cases.

MUM1 is a lymphoid-specific member of the interferon regulatory factor family of transcription factors. Multiple myeloma oncogene 1 is expressed in plasma cells and a minor subset of germinal centre cells. Several studies found MUM1 to be predictive of worse survival rates (3, 9, 10). Also, we have found MUM1 was associated with worse OS and EFS in our study.

Hans *et al* (3) have compared DLBCL sub-groups and survival rates and stated that GCB group was associated to better OS. Whereas several studies have found no survival difference between GCB and non-GCB groups (11, 18, 19). Although the survival rates were higher in GCB group than non-GCB group, but the differences between groups did not reach statistical difference in our study. One reason may be the limited number of cases. The other one may be the contribution of the addition of rituximab to the CHOP regimen. It was shown that high expression of nuclear factor-kappa B (NF-kB) target genes was mainly observed in ABC type but not prominent in GCB type (20). Rituximab, in addition to known exact mechanisms, may suppress the NF-kB signal transduction pathway of tumour cells (21). This may explain the possibility of the lost or weakened prognostic value for the sub-classification of GCB *versus* non-GCB groups. Moreover, some studies have indicated improvement in clinical outcome in non-GCB phenotype and elimination the differences between GCB and non-GCB patients after the addition of rituximab to the chemotherapy regimens (22, 23). So, our findings were consistent with the previous studies outlined above.

Bcl-2 is an anti-apoptotic protein, and Bcl-2 overexpression is detectable in 22%–80% of DLBCL (24). We have evaluated the prognostic value of Bcl-2 expression in DLBCL. Bcl-2 was stained in 18 patients, and of these patients, 12 (66.7%) were positive. In the literature, some studies have investigated the expression of Bcl-2 using immunostains and have found no survival difference (14, 24). Some studies have found that Bcl-2 expression is associated with a significantly worse OS (11, 25, 26). In our study population, we have found no survival difference in terms of Bcl-2 expression. Ki-67 is a protein and serves as a proliferation marker. Higher values indicate lower survival in DLBCL. When we compare Ki-67 index with sub-groups, we found no statistical difference.

Currently, the most effective tool for predicting the outcome of patients with DLBCL is still IPI. In our study, the IPI score predicted OS and EFS when comparing those with low (0–2) *versus* high (3–5) scores. Namely, the IPI was an independent predictor of survival in our study. However, when separately considering the patients with low- or high-IPI scores, there was no survival benefit between GCB and non-GCB phenotypes.

The results of this study are subjected to some limitations. Firstly, this study is a single-centre study with a relatively small sample size, which might underestimate or overestimate the results. Secondly, we could not correlate results with a molecular test such as GEP study.

In conclusion, IHC staining is a widespread method and easy to perform. If IHC markers can be standardized in the future, the risk profiles of the patients will be differentiated clearly, and more accurate treatment schedules will have been established. However, data on the effectiveness of novel therapeutics such as bortezomib in ABC phenotype of DLBCL are accumulating (27, 28). Also, the addition of rituximab on survival benefit should not be ignored. So, more specifically designed prospective studies are needed to externally cross-validate our findings in a larger cohort of DLBCL patients.

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AUTHORS' NOTE

The authors of this paper have no conflict of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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