RESEARCH ARTICLE

Evaluation of BCL-6, CD10, CD138 and MUM-1 Expression in Diffuse Large B-Cell Lymphoma patients: CD138 is a Marker of Poor Prognosis

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Abstract

The diffuse large B-cell lymphoma (DLBCL) encompasses two major groups of tumors with uneven survival outcomes - germinal center B-cell (GCB) and non-germinal center B-cell (non-GCB). In the present study, we investigated the expression of GCB markers (BCL-6 and CD10) and non-GCB markers (CD138 and MUM-1) in an effort to evaluate their prognostic value. Paraffin-embedded tumor biopsies of 46 Jordanian DLBCL patients were analyzed, retrospectively, by immunohistochemistry to investigate the expression of BCL-6, CD10, CD138 and MUM-1. In addition, survival curves were calculated with reference to marker expression, age, sex and nodal involvement. Positive expression of BCL-6, CD10, CD138 and MUM-1 was shown in 78%, 61%, 39% and 91% of the cases, respectively, that of BCL-6 being associated with better overall survival (p = 0.02), whereas positive CD138 was linked with poor overall survival (p = 0.01). The expression of CD10 and MUM-1 had no impact on the overall survival. Among the clinical characteristics studied, diagnosis at an early age, nodal involvement and maleness were associated with a higher overall survival for DLBCL patients. Our results underline the importance of BCL-6 as a marker of better prognosis and CD138 as a marker of poor prognosis for DLBCL patients.

Keywords: Diffuse large B-cell lymphoma - immunohistochemistry - BCL 6 - CD10 - CD138 - MUM 1 - prognosis

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoid malignancy, accounting for more than 30% of adult cases of non-Hodgkin lymphoma (NHL) world-wide (Groves et al., 2000; Hunt and Reichard, 2008). In Jordan, DLBCL accounts for approximately 28% of NHL (Haddadin, 2005). DLBCL represents a heterogeneous group of tumors which could originate *de novo* or by progression of a preexisting tumor such as follicular lymphoma (Pileri et al., 2002). DLBCL patients are commonly treated with the CHOP (Cyclophosphamide, Hydroxydaunorubicin/Adriamycin, Oncovin/Vincristine, Prednisone) chemotherapeutic regimen. The use of rituximab in combination with standard CHOP treatment (R-CHOP) has been shown to increase remission and overall survival (OS) rates among patients (Coiffier et al., 2002).

Patients' clinical characteristics including age, tumor stage, serum lactate dehydrogenase levels, patient performance and nodal involvement, described by the International Prognostic Index are commonly used for the prognostic stratification of DLBCL patients (Lopez et al., 1992; Lossos and Morgensztern, 2006; Magomedova and Vorob'ev, 2008). These variables can be useful as prognostic indicators even in the absence of a clear model relating them to the etiology of the disease. Recently, RNA expression studies have been used to classify DLBCL patients into distinct subgroups with presumed prognostic value (Alizadeh et al., 2000; Lossos et al., 2003; Wright et al., 2003). Accordingly, lymphomas expressing genes characteristic of the germinal center are subgrouped as germinal center B-cells (GCB) and those expressing genes subsequent to the activation of peripheral blood B-cells are subgrouped as non-germinal center B-cells (non-GCB). The GCB subgroup was found to be associated with better overall survival than the non-GCB subgroup (Alizadeh et al., 2000; Lossos et al., 2003; Wright et al., 2003).

The RNA expression studies prompted several groups to look for protein expression markers which could distinguish between the GCB and the non-GCB subgroups (Lossos and Morgenszter, 2006). A number of studies identified BCL-6 and CD10 as markers of the GCB-subgroup and CD138 and MUM-1/IRF4 as markers of the non-GCB subgroup. As such, and assuming the diagnostic value for the GCB/non-GCB classification, these proteins have the potential to be routinely used in diagnostic laboratory settings to predict the clinical

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outcome of patients (Chang et al., 2004; Hans et al., 2004; Paepe and Wolf-Peeters, 2007).

BCL-6 is a zinc-finger transcriptional repressor selectively expressed in germinal center B-cells (Phan and Dalla-Favera, 2004), and has been consistently associated with a better OS (Berglund et al., 2005; Zinzani et al., 2005; Kojima et al., 2006; Sjo et al., 2007).

CD10 is a membrane-bound neutral endopeptidase exclusively expressed in the germinal center cells of lymphoid tissues (Dogan et al., 2000). Several studies reported no prognostic value of this marker (Fabiani et al., 2004; Hans et al., 2004; Oh and Park, 2006). while others reported that the positive expression of CD10 was associated with better OS (Oshima et al., 2001; Hans et al., 2004; Sjo et al., 2007), and poor OS (Uherova et al., 2001; Xu et al., 2001).

CD138, also known as syndecan-1, is a sulphate-rich proteoglycan adhesion molecule usually expressed during B-cells differentiation into plasma cells (Jourdan et al., 1998). Two studies proposed the positive expression of CD138 as a marker of poor prognosis in DLBCL patients (Hoffmann et al., 2005; Oh and Park, 2006).

MUM-1/IRF4 (multiple myeloma oncogene 1/ interferon regulatory factor 4) is a lymphoid specific gene, which is a member of the interferon regulatory factor family of transcription factors (Tsuboi et al., 2000). Different studies show conflicting results regarding the prognostic value of MUM-1, with some reporting poor prognostic value (Braaten et al., 2003; Oh and Park, 2006; Sjo et al., 2007) while others failing to find any significant prognostic value (Chang et al., 2004; Hans et al., 2004; Muris et al., 2006).

In order to investigate the value of BCL-6, CD10, CD138, and MUM-1 as prognostic predictors, we retrospectively examined the expression of these markers in 46 DLBCL patients by means of immunohistochemistry. We also examined the possible correlations between certain clinical presentations (age, sex and nodal involvement) and overall survival.

Materials and Methods

Patients

A total of 46 diffuse large B cell lymphoma patients (25 males and 21 females), diagnosed between 2003 and 2006 with a median age of 56 years (range 16–80) were included in this retrospective study. The patients were identified from the files of the Department of Pathology at the King Abdullah University Hospital in Ramtha, Jordan.

Paraffin-embedded tumor biopsies from the time of diagnosis and a clinical follow-up were available for all patients. The pathology was reviewed by one of us (I.M.) and confirmed to be DLBCL. Histological criteria used for diagnoses and classification of cases were based on morphological examination of paraffin sections and immunophenotyping as described in the World Health Organization (WHO) and the Revised European-American classification of Lymphomas (REAL) (Harris et al., 1994). The DLBCL tumors were classified according to the site of presentation. Patients with a clinically dominant lymph node, as well as those with tumors presenting at the spleen, were considered as primary nodal tumors, whereas patients without nodal involvement or with only a minor nodal involvement together with a clinically major extra-nodal component were considered as extra-nodal. The two groups were compared in terms of clinical characteristics, namely, age, tumor size, serum Alkaline Phosphotase (ALK), serum Lactate Dehydrogenase (LDH), and White Blood Cells count (WBC).

Of the 46 patients, 10 were treated with the standard CHOP chemotherapy, 9 were treated with CHOP in combination with Rituximab (R-CHOP) and 23 patients received other forms of therapy. The median follow up period for all patients (i.e., the time when overall survival is 50%) was 34 months (range 2-38 months). The clinical data for all patients are summarized in Table 1.

Information on patient demographics and survival was retrieved from medical files. Approval for this work was obtained from the University Review Committee for Research on Humans, Faculty of Medicine, Jordan University of Science and Technology.

Immunohistochemical analysis

An immunohistochemical examination was performed on formalin-fixed paraffin sections using the avidin-biotin-peroxidase complex method. Sections were cut to 3 μm thickness, deparaffinized in xylene and rehydrated through graded alcohol in distilled water. Antigen retrieval was done in a commercial buffer (Reveal from

Table 1. Clinical Characteristics of 46 Cases of DLBCL

Characteristic	(n = 46)
Median age (range)	56 (16-80)
Male/female ratio	25/21
Nodal involvement	
Nodal†	25 (54%)
Extranodal†	21 (46%)
Tumor size (cm^3)	
0-5	25 (57%)
6-10	7 (16%)
11-20	3 (7%)
>20	9 (20%)
ALK†, median = 227 IU/L	Range 15-1530 IU/L
Normal	24 (57%)
High	18 (43%)
LDH†, median = 395 IU/L	Range 54-2945 IU/L
Normal	12 (31%)
High	27 (69%)
WBC†, median = 7650 /μl	Range 200-28000 /μl
Low	8 (18%)
Normal	27 (60%)
High	10 (22%)
Survival/death ratio	31/12
Follow up time	
Mean, months (95% CI†)	28.9 (24.4-33.4)
Median, months (95% CI†)	34 (22.8-45.2)
Cumulative survival after 10 months	87%
Cumulative survival after 20 months	83%
Cumulative survival after 30 months	68%

†Nodal, presenting with primary nodal site; Extranodal, presenting with extranodal sites; ALK, Alkaline Phosphatase; LDH, Lactate Dehydrogenase; WBC, White Blood Cells; CI, Confidence interval.

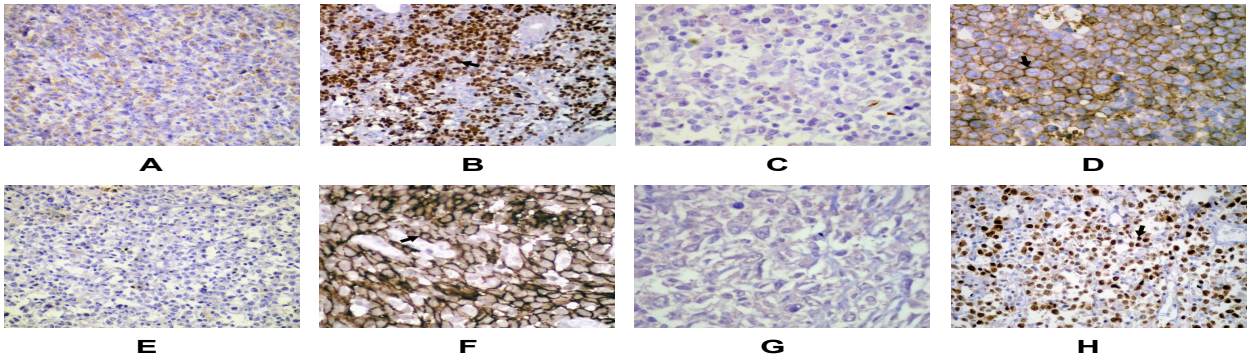

Figure 2. Immunohistochemical Staining of BCL-6, CD10, CD138 and MUM-1. A, Negative BCL-6 expression (Magnification, × 200). B, Positive BCL-6 expression showing diffuse or granular nuclear staining pattern (Magnification, × 400). C, Negative CD10 expression (Magnification, × 400). D, Positive CD10 expression showing diffuse or granular nuclear staining pattern (Magnification, × 400). E, Negative CD138 expression (Magnification, × 100). F, Positive CD138 expression showing a membranous staining pattern (Magnification, × 400). G, Negative MUM-1 expression (Magnification, × 400). H, Positive MUM-1 expression showing a membranous staining pattern (Magnification, × 400).

Biocare) by autoclaving for 7 minutes at 121 ºC and 15 psi atmospheric pressure. After autoclaving, sections were allowed to cool at room temperature for 20 minutes, rinsed thoroughly with distilled water and placed in phosphate buffer saline (PBS). Endogenous peroxidase activity was blocked with hydrogen peroxide and, subsequently, slides were washed with PBS. Immunostaining for the different antibodies was performed on an automated immunostainer (Autostainer XL, LEICA, Germany) according to the manufacturer's protocols. All primary antibodies were incubated for 1 hour at room temperature. Following primary antibody incubation, sections were washed thoroughly with PBS and incubated with biotinylated rabbit anti-mouse antibody (DAKO, Denmark; diluted at 1/200) for 30 minutes at room temperature. Subsequently, sections were washed with PBS and incubated with HRP coupled avidin-biotin complex (DAKO, Denmark) for 30 minutes at room temperature. Following a final wash with PBS, immunoreactivity was visualized with 3, 3-diaminobenzidine (DAB). Finally, sections were briefly counterstained with Mayer's hematoxyline and examined by light microscopy.

Tissues known to express the markers of interest (reactive tonsil) were used as positive controls. For negative controls, sections were treated as described above except the primary antibody was not included in the reaction.

All specimens were analyzed in a semi-quantitative way after a thorough examination of the whole immunostained slide. At least 200 cells were scored in well-preserved areas of each slide. Specimens were classified into 5 groups: 1 (0% to less than 10% positive cells), 2 (10%-25% positive cells), 3 (26%-50% positive cells), 4 (51%-75% positive cells), and 5 (> 75% positive cells), where the percentage is that of tumor cells stained with a given marker. The expression of the investigated markers (BCL-6, MUM-1, CD138, and CD10) was considered positive when the score was 2 or above (i.e., a cutoff value of 10%) (see Figure 1 for staining). In the case of BCL-6 and MUM-1 expression, positivity was scored only when tumor cells exhibited diffuse or granular nuclear staining. CD138 and CD10 usually display a typical membrane staining in tumor cells and thus, the presence of such staining pattern was considered positive when analyzing the expression of these two markers.

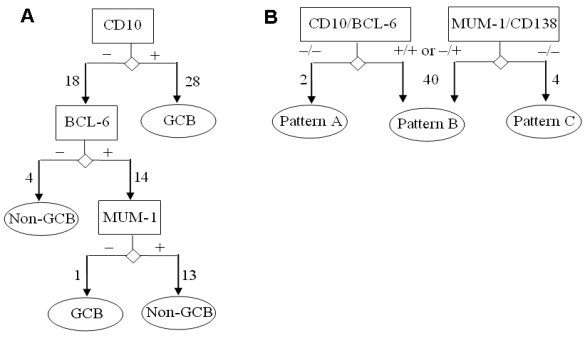

Figure 2. Decision Algorithm for Classifying DLBCL Cases as Described by A, Hans et al. (2004) and B, Chang et al. (2004). (+) indicates cases with positive expression and (–) indicates cases with negative expression.

The DLBCL specimens were subclassified according to the algorithms of Hans et al. (2004) and Chang et al. (2004) (Figure 2). The resultant subgroups were tested for differences in clinical characteristics and OS.

Statistical Analysis

The Mann-Whitney test (Mann and Whitney, 1947) was used to test for clinical differences (age, tumor size, serum ALK concentration, serum LDH concentration, and WBC count) between the nodal and extranodal subgroups, and GCB and non-GCB groups.

The Kaplan-Meier survival curves (Kaplan and Meier, 1958) were used to represent the overall survival (OS) distributions. Overall survival differences for the different markers and clinical presentations were analyzed using the log rank test (Mantel, 1966), and the Breslow test (Breslow, 1970). Overall survival was defined as the period from the time of diagnosis to death from any cause or the last contact. The log rank method gives equal weights to all time intervals thus individuals surviving to late stages influence the statistics more than individuals who drop off the analysis early. In contrast, the Breslow method gives each time interval a weight that is proportional to the number of individuals surviving that interval, thus early stages contribute more to the statistic than latter stages, which usually have few individuals. Categories

with less than five cases were not included. Multivariate analysis was not feasible in the present study due to the fact the data does not fit the proportionality assumption. The significance level was set to p≤5%. The Statistical Package for the Social Sciences program was used for data analysis (for Windows 13.0, SPSS Inc., USA).

Results

Clinical data

Out of the forty-six DLBCL patients, twenty-five patients had a primary nodal involvement (nodal subgroup), whereas twenty-one patients were presented with two or more extranodal sites (extranodal subgroup). The two subgroups did not exhibit any significant differences with respect to clinical parameters (age, tumor size, serum ALK concentration, serum LDH concentration, WBC count) as shown in Table 2.

Table 2. Stratified Survival Analysis According to Nodal Involvement.

Variable	Category	N (deaths)	Logrank (pvalue)	Breslow (pvalue)
Stratified survival analysis according to nodal involvement.				
Nodal patients only (N=24)				
Gender	Male	14 (1)	3.4 (0.06)	2.1 (0.1)
	Female	10 (3)		
BCL6	Negative	6 (2)	1.5 (0.2)	0.01 (0.9)
	Positive	18 (2)		
CD138	Negative	14 (3)	0.2 (0.7)	0.8 (0.4)
	Positive	10 (1)		
Extranodal patients only (N= 19)				
Gender	Male	9 (2)	1.5 (0.2)	3.5 (0.06)
	Female	10 (6)		
BCL6	Negative	3 (3)	3.8 (0.05)	1.3 (0.3)
	Positive	16 (5)		
CD138	Negative	11 (3)	8.9 (0.003)	8.8(0.003)
	Positive	8 (5)		
Stratified survival analysis according to gender.				
Male patients only (N=23)				
Nodality	Nodal	14 (1)	2.0 (0.2)	1.5 (0.2)
	Extranodal	9 (2)		
BCL6	Negative	3 (1)	7.0 (0.008)	7.0(0.008)
	Positive	20 (2)		
CD138	Negative	14 (3)	0.6 (0.4)	0.6 (0.4)
	Positive	9 (0)		
Female patients only (N=20)				
Nodality	Nodal	10 (3)	1.1 (0.3)	2.7 (0.1)
	Extranodal	10 (6)		
BCL6	Negative	6 (4)	0.5 (0.5)	0.03 (0.9)
	Positive	14 (5)		
CD138	Negative	11 (3)	9.4 (0.002)	8.5(0.004)
	Positive	9 (6)		
Stratified survival analysis according to way of treatment.				
NonTreated patients only (N=22)				
Gender	Male	13 (1)	2.4 (0.1)	2.2 (0.1)
	Female	9 (3)		
Nodality	Nodal	11 (1)	1.9 (0.2)	1.9 (0.2)
	Extranodal	11 (3)		
BCL6	Negative	2 (1)	2.7 (0.1)	1.1 (0.3)
	Positive	20 (4)		
CD138	Negative	13 (2)	2.7 (0.1)	2.6 (0.1)
	Positive	9 (2)		
Treated patients only (N=19)				
Gender	Male	9 (2)	2.9 (0.09)	2.8 (0.1)
	Female	10 (5)		
Nodality	Nodal	12 (3)	0.1 (0.7)	1 (0.3)
	Extranodal	7 (4)		
BCL6	Negative	7 (4)	2.4 (0.1)	0.4 (0.5)
	Positive	12 (3)		
CD138	Negative	12 (4)	3.7 (0.05)	5.1 (0.02)
	Positive	7 (3)		

Protein expression

Results of the immunohistochemical staining of markers for both nodal and extranodal subgroups are summarized in Table 3. Positive expression of CD10 was seen in 61% (28 out of 46) of the cases, BCL-6 in 78% (36 out of 46), MUM-1 in 91% (42 out of 46) and CD138 in 39% (18 out of 46). Based on the algorithm of Hans et al. (2004) 63% (29 of 46) of patients were subgrouped as GCB and 37% (17 of 46) of patients were subgrouped as non-GCB, as shown in Figure 1A. In addition, we used the classification system suggested by Chang et al. (2004) to further subgroup the cases. Accordingly, 4% (2 out of 46) of patients were subclassified as pattern A, 87% (40 out of 46) as pattern B, and 9% (4 out of 46) were of pattern C, as shown in Figure 1B. A summary of the subgrouping of all cases is shown in Table 4.

Prognostic value and survival analysis

We investigated the overall survival of 43 cases of DLBCL patients (3 subjects were excluded from the

Table 3. Testing for Differences in Clinical Parameters between Patients Classified According to Nodal Involvement and the Hans et al. (2004) Algorithm.

Variables	Age	Tumor Size	ALK†	LDH†	WBC†
Nodal involvement					
Mann-Whitney's U	215.5	161.5	214.5	175.0	248.5
p-value	0.6	0.07	0.9	0.8	1.0
Hans grouping‡					
Mann-Whitney's U	174.5	198.0	170.5	128.0	173.5
p-value	0.2	0.5	0.3	0.1	0.2

†ALK, Alkaline Phosphotase; LDH, Lactate Dehydrogenase; WBC, White Blood Cells. ‡Hans grouping was based on the 10% cutoff value for expression.

Table 4. Overall Survival According to Grouping Methods of Hans et al. (2004) and Chang et al. (2004) Based on Different Cutoff Values.

Variables	Cases	Log Rank		Breslow	
Categories	N (Deaths)	χ^2	p-value	χ^2	p-value
Hans grouping at 10% cutoff					
GCB	27 (7)	1.5	0.2	1.1	0.3
Non-GCB	16 (5)				
Hans grouping at 25% cutoff					
GCB	17 (4)	1	0.3	1	0.3
Non-GCB	26 (8)				
Chang grouping at 10% cutoff					
Pattern A	2 (0)	N/A†	N/A†	N/A†	N/A†
Pattern B or C	41 (12)				
Chang grouping at 25% cutoff					
Pattern A	8 (2)	0.1	0.7	1.2	0.3
Pattern B or C	30 (8)				

†N/A, not applicable.

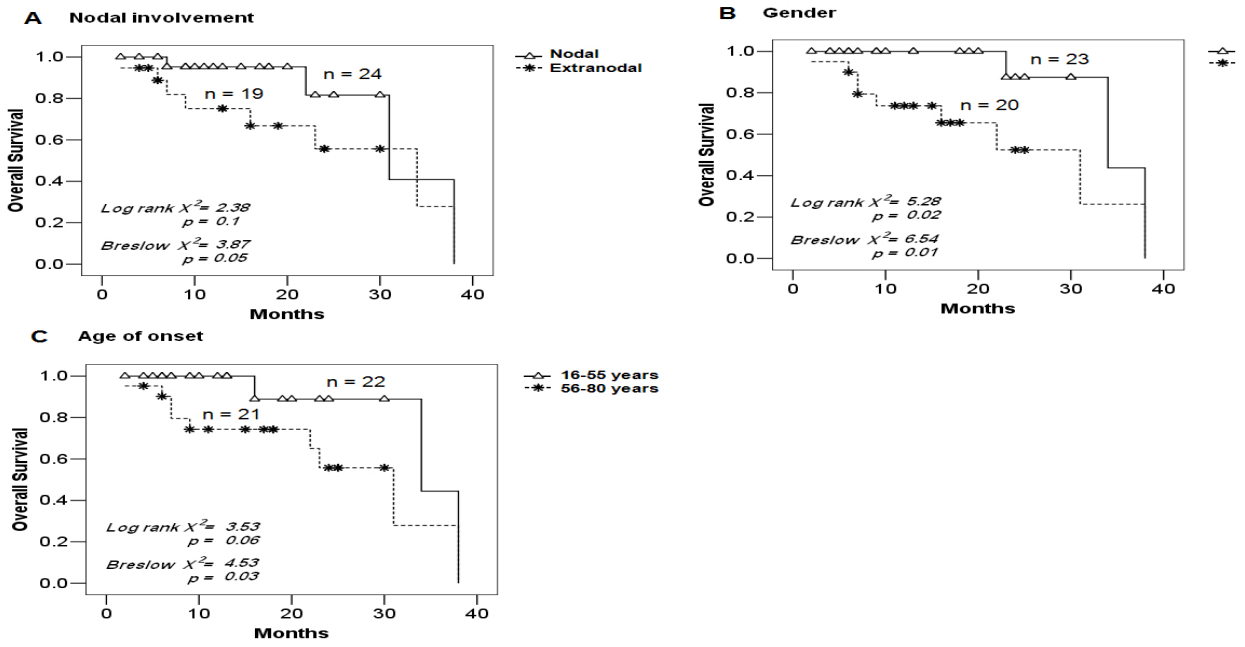

Figure 3. Kaplan-Meier Survival Analysis in DLBCL According. A) Nodal involvement, B) Gender and C) Age. Patients presenting with primarily nodal sites have a relatively better overall survival when compared to the ones presenting with primarily extranodal sites (A). Males with DLBCL show a significantly better overall survival when compared to females with the disease (B). Older patients display a relatively worse overall survival (C).

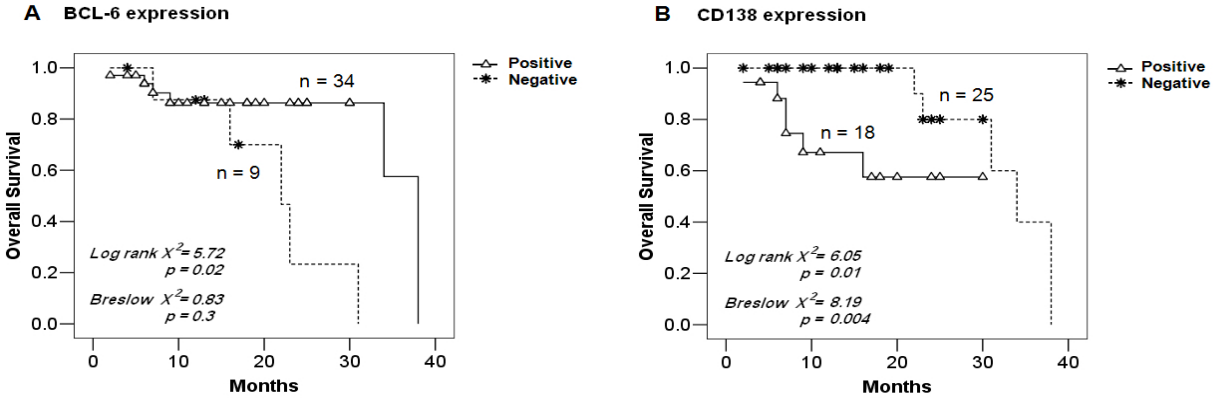

Figure 4. Kaplan-Meier Survival Analysis in DLBCL According. A) BCL-6 expression and B) CD138 expression. Patients with positive BCL-6 expression have a significantly better overall survival (A). Patients with positive CD138 expression have a significantly worse overall survival (B).

analysis for lack of follow-up data). Variables analyzed included nodal involvement, gender, age of onset, marker expression, and GCB vs. non-GCB classification.

Patients with primarily nodal involvement had a marginally better overall survival compared to patients with extranodal involvement according to the Breslow test (χ^2 = 3.9, p = 0.05, Figure 3A) but not the log rank (χ^2 = 2.4, p = 0.1). Males with DLBCL had a significantly better overall survival when compared to female patients (Log rank χ^2 = 5.3, p = 0.02, Breslow χ^2 = 6.5, p = 0.01, Figure 3B). When using the median age of 56 years as a cutoff point to categorize the patients by age, our results suggest that older patients have a relatively worse overall survival than younger patients according to the Breslow test (χ^2 = 4.5, p = 0.03, Figure 3C), but marginally so according to the log rank (χ^2 = 3.5, p = 0.06). Using the IPI standard age cutoff point of 60 years resulted in the loss of statistical significance (Log rank χ^2 = 3.4, p = 0.07, Breslow χ^2 = 2.8 p = 0.09).

The positive expression of BCL-6 was associated with better survival according to the log rank test (χ^2 = 5.7, p = 0.02, Figure 4A), but not the Breslow test (χ^2 = 0.8, p = 0.4).

The expression of CD10 was not associated with any difference in OS (Log rank χ^2 = 1.5, p = 0. 2, Breslow χ^2 = 1.0, p = 0.3).

Positive expression of CD138 was found to be associated with a significantly poor overall survival (Log rank χ^2 = 6.1, p = 0.01, Breslow χ^2 = 8.2, p = 0.004, Figure 4B).

Thirty-nine out of forty-three MUM-1 cases displayed positive expression and thus no statistical analysis was feasible.

Subgrouping of cases into GCB and non-GCB did not result in any statistical significant difference in overall survival (Log rank χ^2 = 1.5, p = 0.2, Breslow χ^2 = 1.1, p = 0.3). In addition, this subgrouping did not result in any statistically significant difference in respect to clinical

parameters (age, tumor size, serum ALK concentration, serum LDH concentration, WBC count) as shown in Table 3.

Since forty out of forty-three subjects were classified as pattern B in Chang's classification, statistical analysis was not feasible. Stratified survival analysis was also used to investigate bias in our test. Stratification was performed according to nodal involvement, gender, and treatment.

Discussion

In the present study, we used immunohistochemistry to examine the expression of BCL-6, CD10, CD138 and MUM-1 in forty-six Jordanian DLBCL patients and evaluated the possible use of these markers as prognostic markers, alongside other presentation variables such as age, gender, and nodal involvement.

Subjects with nodal presentation had a significantly better overall survival than those with extranodal presentation according to the Breslow test ($p = 0.05$) but not the log rank test ($p = 0.1$). Figure 3A shows that after the 30th month interval there was an overlap in the survival of the two groups which must have depressed the log rank test value and raised its corresponding p-value (the long rank test is strongly affected by occurrences in late time intervals since it weights all time intervals equally, while the Breslow test weights each interval in proportion to the number of surviving individuals; in this case only 4 individuals survived beyond the 30th month). Previous studies reported similar results associating primary nodal involvement with increased OS (Coiffier et al., 2002; Colomo et al., 2003; Hoffmann et al., 2005) , while other studies did not show a statistically significant relationship (Xu et al., 2001; Chang et al., 2004; Liu et al., 2008).

No statistically significant differences were found in the clinical presentations (age, tumor size, serum ALK concentration, serum LDH concentration, WBC count) between the nodal and the extranodal groups (Table 3).

Male DLBCL patients displayed a significant increase in overall survival when compared to female patients (Figure 3B). As far as we know, this is the first time a gender-related difference in DLBCL prognosis is reported. It is possible that this difference may not have a biological significance, but is rather related to gender differences in adherence to treatment, number of follow-up visits, and other health care-related issues in Jordan.

Furthermore, patients with late disease onset showed a significant decrease in overall survival according to the Breslow test ($p = 0.03$), but rather a marginally significant decrease in overall survival according to the log rank test ($p = 0.06$). This finding is consistent with previous studies (Colomo et al., 2003; Chang et al., 2004; Liu et al., 2008). It is not surprising for older patients to have a reduced survival compared to younger patients given the expected decline in their general health and performance, on average.

In the present study, positive expression of BCL-6 was seen in 78% of the cases. This falls within the range of reported expression frequencies in studies employing the same antibody source and applying the same 10% cutoff value used in this study: 63% (Lossos et al., 2001), 79% (Braaten et al., 2003), and 97% (Linderoth et al., 2003). A study by Harada et al. (1999) also reported a 92% expression frequency; however, the antibody used was not specified.

Our results indicate a significant increase in long-term survival in Jordanian DLBCL patients with positive BCL-6 expression detected by the PG-B6p antibody (DAKO) and using a 10% cutoff value for scoring positive expression according to the log rank test ($p = 0.02$) but not the Breslow test ($p = 0.4$). This mixed result reflects an observation that the subjects with positive expression of BCL-6 showed improved survival in the latter stage of the disease (Figure 4A). We compared this finding with eighteen previous studies which examined the prognostic value of BCL6 expression for DLBCL patients. Of these studies, only Barrans et al. (2002) reported a statistically significant reduction of OS in association with positive BCL6 expression in DLBCL patients. Barrans et al.'s deviant result might be related to their use of the anti-BCL6 n-3 antibody, in contrast to the majority of the other studies which used the same PG-B6p antibody we employed in the present study (e.g. Losos et al., 2001; Colomo et al., 2003; Muris et al., 2006; Liu et al., 2008).

Aside from Barrans et al. (2002), seven out of the seventeen remaining studies of BCL-6 expression in DLBCL patients reported a significant association between positive expression of BCL-6 and improved OS (e.g. Harada et al., 1999; Braaten et al., 2003; Berglund et al., 2005; Sjo et al., 2007), whereas nine studies did not report any significant difference in OS between positive and negative BCL-6 expression in DLBCL patients (e.g. Colomo et al., 2003; Kojima et al., 2006; Lin et al., 2006; Muris et al., 2006). A study by Winter et al (2006), reported a significantly increased OS in DLBCL patients with positive BCL-6 expression that were treated with the CHOP regimen, but no difference in OS related to BCL-6 expression in patients treated with R-CHOP.

Similar to the findings of our study, almost all studies that set the expression cutoff value to 10% reported increased OS in association with positive BCL-6 expression (Harada et al., 1999; Lossos et al., 2001; Braaten et al., 2003). The sole exception is Linderoth et al. (2003) who only scored four subjects with negative BCL-6 expression in their sample—too few for meaningful statistical significance testing. Furthermore, two of the studies that reported a statistically significant association between positive expression of BCL-6 and improved survival, Harrada et al. (1999) and Bratten et al. (2003) scored only six and seven patients with positive BCL-6 expression, respectively, indicating that the effect size of this relationship is large.

Interestingly, when we reanalyzed our data using a cutoff value of 25% for scoring BCL-6 expression, no significant difference was found (log rank $\chi^2 = 1.4$, $p = 0.2$; as compared to a log rank value of 5.7 and p value of 0.02 using the 10% cutoff). This finding is consistent with eight studies out of ten that used a scoring cutoff point in the range of 20-30% failing to find a significant change in OS in relation to BCL-6 expression. It is important to note that this loss of statistical significance in our study was not due to the 25% cutoff resulting in an unbalanced

design. In fact, the 25% cutoff resulted in a more balanced design (16 +ve vs. 26 –ve) than the 10% cutoff (33 +ve vs. 9–ve). This suggests the prognosis of individuals with less than 10% expression of BCL-6 in their tumor tissue is indeed different from that of individuals with more than 10% expression, but that there is little difference between the prognosis of patients with 10-25% BCL-6 expression and that of patients with more than 25% expression.

In the present study, positive expression of CD10 was scored in 61% of the cases, using the 10% cutoff value. Previous studies that applied the same cutoff value of 10% reported a lower frequency of positive expression of CD10 [19% (Braaten et al., 2003), 34% (Fabiani et al., 2004), 43% (Xu et al., 2001), and 51% (Linderoth et al., 2003)]

Other studies, using the 20%, 25%, 30% and 50% cutoff values reported a wide range of CD10 expression in DLBCL tumors (2 to 92%) (e.g. Harada et al., 1999; Ohshima et al., 2001; Pileri et al., 2003; Camilleri-Broet et al., 2006).

The present study found no significant change in OS of Jordanian DLBCL patients in association with CD10 expression using the 56C6 antibody and a 10% cutoff value (log rank p = 0.2). We compared this finding with seventeen studies which examined the prognostic value of CD10 expression for DLBCL patients.

Of these studies, eight of them reported a significant association between positive expression of CD10 and improved OS in DLBCL patients (e.g. Tzankov et al., 2003; Chang et al., 2004; Hans et al., 2004; Hoffmann et al., 2005), seven studies did not report any significant difference (e.g. Braaten et al., 2003; Fabiani et al., 2004; Oh and Park, 2006; Liu et al., 2008), and two studies reported a statistically significant reduction of OS in association with positive BCL6 expression in DLBCL patients (Uherova et al., 2001; Xu et al., 2001).

It is worth noting that latter two studies, Uherova et al. (2001) and Xu et al. (2001) used flow cytometry to detect CD10 expression, while all of the other studies relied on the same immunohistochemical protocols used in our study. Furthermore, Uherova et al. (2001) and Xu et al. (2001) used the W8E7 antibody for the detection of CD10 expression which no other study reportedly used. The majority of the seventeen studies reported using the same 56C6 antibody employed in our study (e.g. Linderoth et al., 2003; Chang et al., 2004; Hans et al., 2004; Oh and Park, 2006).

Ohshima et al. (2001) and Hoffman et al. (2005) give Novocastra as their CD10 antibody source, while Muris et al. (2006) give Monosan as their CD10 antibody sources, however both companies has more than one CD10 clone available (including 56C6) and the three studies do not specify which of the clones were used. Of the CD10 immunohistochemical studies only Tzankov et al. (2003) specified a CD10 antibody other than 56C6, viz. the SS2/36 antibody.

Some of the studies that did not find a statistically significant association between CD10 expression and DLBCL survival relied on a few individuals with positive CD10 expression to make the comparison (Braaten et al., 2003; Camilleri-Broet et al., 2006). However, other studies boasted respectable sample sizes of CD10 positive patients (ranging from 27 to 63) (Colomo et al., 2003; Linderoth et al., 2003; Fabiani et al., 2004; Liu et al., 2008) and still did not find a significant association between CD10 expression and DLBCL survival.

Interestingly, no patterns of difference in the use of antibodies, the choice of expression cutoff value, or sample size could be discerned between the studies that found a positive prognostic value for CD10 expression and those that failed to do so. It is possible that other factors such as the particular genetics of the different patient populations could be involved.

In the present study, CD138 is shown to be expressed in 39% of the cases studied, using a cutoff value of 10%. This frequency is higher than these reported in previous studies applying the same cutoff value of 10% [0% (Braaten et al., 2003), 0% (Pileri et al., 2003), 2% (Linderoth et al., 2003]. However, several other studies applying higher cutoff value reported expression in higher frequencies [7% (Tzankov et al., 2003), 15% (Hoffman et al., 2005), 16% (Oh and Park, 2006)]

In the present study, we report a significant decrease of OS in Jordanian DLBCL patients with positive CD138 expression using the MI15 antibody (DAKO) and with a cutoff value set at 10% (log rank p = 0.01, Figure 4B). We compared this finding with fourteen studies which examined the prognostic value of CD138 expression for DLBCL patients. Of these studies, only two reported a statistically significant reduction of OS in association with positive CD138 expression in DLBCL patients (Hoffmann et al., 2005; Oh and Park, 2006). These two studies used the MI15 antibody as in our study, but conversely set the expression scoring cutoff value to 30% (Oh and Park, 2006) and 50% (Hoffmann et al., 2005).

None of the twelve other studies reported a statistically significant difference in DLBCL prognosis in association with CD138 expression. It should be noted that eleven out of these twelve studies scored seven or fewer patients with positive CD138 expression, thus limiting the possibility of a meaningful statistical analysis. Saez et al. (2004) scored 15 patients with positive CD138 using the MI15 antibody and still failed to find statistical significance, however they used an expression cutoff value of 80% which complicates the comparison with our study.

Although the survival according to CD138 expression in this study was not affected by the course of treatment, it is noteworthy to mention that Females and patients with extranodal involvement showed more significant difference in OS compared to males and patients with primary nodal involvement.

MUM-1 expression was scored in 91% of the cases studied, using the 10% cutoff value. A high frequency of MUM-1 expression in DLBCL was also reported by Braaten et al. (2003), the only other study that used the 10% cutoff value.

The high frequency of MUM-1 expression in our sample left us with only three individuals with negative expression (out of 43 patients with survival data), too low for a meaningful statistical comparison.

Sixteen previous studies examined the prognostic value of MUM-1 expression for DLBCL patients. Of these studies, six reported significant association between

positive MUM-1 expression and decreased OS (Chang et al., 2004; Hans et al., 2004; Lin et al., 2006; Muris et al., 2006; Hallermann et al., 2007; Liu et al., 2008), while ten failed to find a significant difference in OS between patients with positive and negative MUM-1 expression (e.g. Berglund et al., 2005; Kojima et al., 2006; Oh and Park, 2006; Sjo et al., 2007). Three out of these ten studies (Braaten et al., 2003; Camilleri-Broet et al., 2006; Lin et al., 2006) scored seven or fewer negative samples for MUM-1 expression, severely reducing the statistical power of testing in these studies. Other studies, however, had considerable sample sizes of both MUM-1 positive and MUM-1 negative DLBCL patients and still failed to detect a significant difference in the survival of the two groups (Colomo et al., 2003; Saez et al., 2004; Berglund et al., 2005; Hoffmann et al., 2005; Sjo et al., 2007).

As previously observed for studies of CD10 expression, no patterns of difference in the use of antibodies, the choice of expression cutoff value, or sample size could be discerned between the studies that found a negative prognostic value for MUM-1 expression and those that failed to do so, thus suggesting that other factors such as particular population genetics might be at play.

In the present study, the percentages of GCB vs. non-GCB subgroups were found to be 63% (29 patients) and 37% (17 patients), respectively. Previous reports (Hans et al., 2004; Berglund et al., 2005; Haarer et al., 2006) indicate that DLBCL patients of the GCB subgroups are associated with a better clinical outcome; however, we were not able to detect any such statistically significant difference. It is worth noting that using a different cutoff value of 25% instead of 10% will change the ratio between these subgroups, as shown in Table 5. Still, no statistically significant difference in OS was detected using the classification based on the 25% cutoff.

When the differences in clinical parameters were tested between the GCB/non-GCB groups using a cutoff value of 10%, no statistically significant differences were found. However, using a cutoff value of 25%, the GCB group had significant lower levels of ALK (Mann-Whitney's $U = 130.5$, $p = 0.05$) and younger patients (Mann-Whitney's $U = 142$, $p = 0.02$). Previous studies that used a cutoff value of 30% failed to report clinical differences between the GCB/non-GCB groups in terms of age, sex or LDH (Hans et al., 2004; Kojima et al., 2006; Oh and Park, 2006).

Classification of our samples according to the Chang et al. (2004) using a cutoff value of 10% was not feasible for statistical analysis. Using the 25% cutoff value did not result in any significant difference in OS. However, cases of the pattern A showed statistically significant lower levels of ALK (Mann-Whitney's $U = 54.5$, $p = 0.02$).

Studies of this kind often show discrepancies in results due to idiosyncrasies of sample size, unbalanced ratio between subgroups, treatment regimens, immunohistochemical protocols, choice of antibodies, scoring criteria, inter- and intraobserver differences, and, possibly, the genetics of populations sample. Nevertheless, we believe our study, taken in conjunction with other immunohistochemical studies, is useful in highlighting the importance of BCL-6 and CD138 as markers of better and poor prognosis, respectively. In addition, our results did not demonstrate any prognostic value for CD10 and MUM-1. We also believe that the choice of the cutoff value (i.e., 10% vs. 25% in the present study) results in a striking difference in the interpretation of the data and the conclusions reached such as the case for BCL-6 and CD138. Confinement to nodal sites, male patients and early age of diagnosis are among the clinical characteristics which correlate with better outcome for DLBCL patients. Future work will involve a more comprehensive analysis of BCL-6 and CD138 expression in Jordanian DLBCL patients to better understand their role in the initiation and progression of lymphomas and thus better management of DLBCL patients.

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