Prognostic value of immunohistochemical classification of diffuse large B-cell lymphoma into germinal center B-cell and non-germinal center B-cell subtypes

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ABSTRACT

الأهداف: استخدام البروتينات المتعلقة بوجود المركز الجنيني (GCB) في إيجاد تقسيم تكهني أمثل لمرضى الأورام الليمفاوية المنتشرة ذات الخلية الكبيرة (DLBCL) و دراسة العلاقة بين هذه البروتينات وبين المعايير الإكلينيكية الثابتة.

الطريقة: تم إجراء هذه الدراسة على 30 بلوك شمعي نسيجي من مرضي الأورام الليمفاوية المنتشرة ذات الخلية الكبيرة DLBCL وذلك خلال الفترة من أبريل 2004 حتى يناير 2007 في مستشفيات جامعة عين شمس و المعهد القومي للأورام – القاهرة – مصر. تلقى جميع المرضى الانثراسيكلين و لم يتلقى أي واحد منهم معالجة مناعية بالريتكسامب. تم إجراء التفاعل الكيميائي المناعي النسيجي للاستدلال على CD10, BCL6, MUM1/IRF4 على كل حالة في هذه الدراسة

النتائج: تم تقسيم المرضى إلى مجموعة تحتوي على مركز جنيني GCB 17 مريض، ومجموعة لا تحتوي على مركز جنيني (13 مريض). هنالك علاقة ظاهرة إحصائياً بين النمط الظاهري للمجموعة التي لا تحتوي على مركز جنيني و مستوى الأداء PS>1، ارتفاع مستوى بروتين LDH، و مستوى IPI المتقدم، ونتائج المرضى السيئة. كما ارتبط خطر الوفاة مع النمط الظاهري للمجموعة التي لا تحتوي على مركز جنيني، و ارتفاع مستوى LDH، و مستوى الأداء PS>1. كان متوسط فترة العيش 46.9 شهر في مجموعة أ مقارنة مع 19.6 شهر في مجموعة ب (HR=3.30; 95% CI=0.52-21.10). باستخدام تحليل كوكس الانحداري المتغير، أصبح النمط الظاهري الذي لا يحتوي على مركز جنيني العامل المنبئ (HR=6.07; 95% CI=1.6-22.9; p=0.008).

خاتمة: إن تقسيم الأورام الليمفاوية المنتشرة ذات الخلية الكبيرة DLBCL إلى نوع ذو خليه يحتوي على مركز جنيني وآخر بدون مركز جنيني مما يؤدي إلى التعرف بدقه أكثر على المرضى ذوي العوامل السيئة وإعطائهم نوعيه أكثر شده من العلاج المعتاد.

Objectives: To study the expression of germinal center B-cell (GCB)/activated B-cell like-related proteins to get optimal stratification of diffuse large B-cell lymphoma (DLBCL) patients, and correlate this with the established clinical and laboratory parameters.

Methods: This study was conducted retrospectively on 30 archival paraffin tissue blocks of DLBCL. All patients were diagnosed between April 2004 and January 2007 at Ain Shams University Hospital and National Cancer Institute, Cairo, Egypt. All patients received anthracycline-based regimens, and none of them received rituximab immunotherapy. Each case included in this study was investigated by immunohistochemical reaction for multiple myeloma-1/interferon regulatory factor-4, B-cell/lymphoma 6, and cluster of differentiation10 monoclonal antibodies.

Results: Patients were classified as GCB group (17 patients) and non-GCB group (13 patients). We found a statistically significant association between non-GCB phenotype and performance status (PS) >1, high lactate dehydrogenase (LDH) level, advanced international prognostic index (IPI), and poor patient outcome. Non-GCB phenotype, high LDH level, and PS>1 were all associated with increased mortality risk. The median survival time was 46.9 months in group A compared to 19.6 months in group B (hazard ratio[HR]=3.30; 95% confidence interval [CI]=0.52-21.10). Using multivariate Cox regression analysis, non-GCB phenotype was found to be the most predicting factor (HR=6.07; 95% CI=1.6-22.9; p=0.008).

Conclusion: The subclassification of DLBCL into GCB and non-GCB groups using immunohistochemistry may be useful for identifying those patients whose prognosis is so poor that more aggressive therapy can be given at the time of diagnosis.

Saudi Med J 2010; Vol. 31 (2): 135-141

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Received 25th August 2009. Accepted 5th January 2010.

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Diffuse large B-cell lymphoma (DLBCL) is the most common type of aggressive non-Hodgkin's lymphoma (NHL), and represents approximately 30-40% of adult NHL. It is a heterogeneous group of tumors varying in immunophenotype, cytogenetics, and clinical features.[1] According to the WHO classification, DLBCL's are defined as diffuse proliferations of large neoplastic mature B-cells that include various morphological subtypes; centroblastic, immunoblastic, T-cell/histiocyte-rich, and anaplastic.[2] The International Prognostic Index (IPI) is still one of the most important tools to predict response to treatment for aggressive NHL, and to classify patients into subgroups with distinctly different prognosis. However, even within these IPI risk groups, a substantial variability in outcome has been observed. Thus, finding new tools to better classify DLBCL patients into different prognostic subgroups is important.[3] Gene expression profiling studies of DLBCL have led to the discovery of previously unrecognized 2 molecularly distinct subtypes; one with gene expression patterns demonstrated by germinal center B-cells, which is germinal center B-cell-like (GCB) DLBCL, and the other with expression of genes that are induced during activation of peripheral blood B-cells (post-germinal), which is activated B-cell-like (ABC) DLBCL, and is also called non-GCB and post-germinal DLBCL.[4] Antigens that are differentially expressed at the GCB and post-germinal center stages of B-cell differentiation are cluster of differentiation (CD)10, BCL6 (B-cell/lymphoma 6), and MUM1/IRF4 (multiple myeloma-1/interferon regulatory factor-4).[2] The CD10 is a membrane-associated neutral endopeptidase, and it has a restricted expression in the GCB cells of reactive lymphoid tissues.[5] The BCL6 is a zinc-finger protein that acts as a transcriptional repressor, and is necessary for GCB formation.[6,7] The MUM1/IRF4 is a lymphoid-specific member of the interferone regulatory factor of transcription factors. The protein is normally expressed in plasma cells and a minor subset of GCB cells.[8] In deoxyribonucleic acid (DNA) microarray studies, messenger ribonucleic acid (mRNA) expression of CD10 and BCL6 is suggested to be correlated with GCB phenotype, while MUM1/TRF41 mRNA expression is associated with non-GCB phenotype.[9] However, gene expression profiling analysis is not easily incorporated in routine practice as it depends on the availability of frozen tissue and sophisticated laboratory and statistical methods.[3] Hans et al[10] reported that the immunohistochemistry staining pattern for CD10, BCL6, and MUM/IRF4 may be used to classify DLBCL into GCB and non-GCB groups that correlate prognostically with the groups defined by gene expression profiling. Though several researchers demonstrated that GCB DLBCL patients were associated with better survival,[11-14] yet not all studies confirm this prognostic advantage.[3]

The aim of this work is to study the expression of GCB/ABC-related proteins to get optimal stratification of DLBCL patients into prognostically favorable and unfavorable subgroups, and correlate this with the established clinical and laboratory parameters.

Methods. This study was conducted retrospectively between April 2004 and January 2007 at Ain Shams University Hospital and National Cancer Institute, Cairo, Egypt on 30 existing archival paraffin tissue blocks of DLBCL with follow up period not less than 24 months for surviving patients (range: one-58 months). The study protocol was approved by the Ain Shams Medical Research Ethical Committee. Inclusion in the study was solely based on the availability of clinical information and histological material. Data for clinical and laboratory assessment were collected from the medical records of the patients. Data were recorded in a manner that individuals cannot be identified. All patients received anthracycline-based regimens. Most patients (23) received CHOP regimen (cyclophosphamide, doxorubicin, vincristine, and prednisone) for 4-8 cycles, while 7 patients with past history of cardiac troubles and reduced ejection fraction measured by echocardiography received CNOP regimen (cyclophosphamide, mitoxantrone, vincristine and prednisone). None of the studied patients received rituximab immunotherapy as part of the chemotherapy protocol (the use of immunotherapy was not routinely incorporated in the treatment protocol during this period due to its high cost). Sixteen patients (53%) received involved field radiotherapy (IFRT) after chemotherapy. Patients were classified according to IPI score (the International Non-Hodgkin's Lymphoma Prognostic Factors Project)[15] into 2 groups: low and low-intermediate risk as one group, and intermediate-high and high risk patients as the other group. Performance status (PS) was assessed according to the Eastern Cooperative Oncology Group scale (ECOG).[16] Response was evaluated using the International Working Group (IWG) guidelines for response criteria for lymphoma.[17] These criteria were based on the reduction in the size of the enlarged lymph node as measured by CT scan, and the extent of bone marrow involvement that was determined by bone marrow aspirate and biopsy. Single-photon emission computed tomography gallium scans were used as an adjunct for assessment of the response. Flow cytometry, cytogenetic, and molecular studies were not included in response definitions. Accordingly, patients were subdivided into 2 groups. Good outcome group included patients with complete response (CR) and partial response (PR), while bad outcome group

included patients with stable disease (SD), progressive disease (PD), relapse, or those who died.

Histopathology and immunohistochemistry. Archival lymph node biopsies was independently examined by 3 experienced pathologists who were blinded to the clinicopathological data of the tumor, and to the initial score of other observers, and a high level of concordance (90%) was achieved. In case of disagreement, the slides were reviewed and a consensus view was achieved. Because the staining pattern sometimes varied within the same tumor, the final score was based on the dominant pattern. A diagnosis of DLBCL was ensured according to the standard diagnostic criteria detailed in the WHO classification for NHL, which includes classic histological features and tumor cell immunoreactivity to the documented B-cell markers.[18] Each DLBCL case included in the study was tested immunohistochemically for expression of MUM1/TRF41, BCL6, and CD10. Sections 4 µm thick were cut and mounted on poly-lysine coated slides. Deparaffinized sections were treated by antigen heat retrieval in 50 mmol/L Tris-HCL/2 mmol/L EDTA buffer, pH 9.0 in a microwave oven for 30 minutes. After autoclave pretreatment, sections were allowed to cool down at room temperature, and were immersed in hydrogen peroxide in absolute methanol to inactivate endogenous peroxide. Indirect immunoperoxidase staining was carried out according to standard protocol. Briefly, slides were incubated in 2 separate runs with mouse monoclonal antibodies for MUM1/TRF41 (DakoCytomation, Glostrup, Denmark); BCL6 (Lab Vision, Fremont, CA., USA), CD10 (Lab Vision, Fremont, CA., USA) at room temperature for 60 minutes, 3, 3-diaminobenzidine/ H2O2 (DakoCytomation, Glostrup, Denmark) was used as a chromogen and hematoxylin as a counter stain. Visualizations were performed using diaminobenzidine (DAB) as chromogen with non-neoplastic lymphoid tissues serving as positive control for all the antibodies tested.[1] Positivity of MUM1/TRF41 and BCL6 was demonstrated as brown nuclear staining, whereas positivity of CD10 was demonstrated as brown membranous staining. Tumor immunoreactivity was evaluated semi-quantitatively by 2 or 3 independent observers. The MUM1/TRF41, BCL6, and CD10 were considered positive when more than 30% of the tumor cells were positive.[3] To validate our data, we applied the 3 GCB/ABC-related markers, based on the algorithm published by Hans et al,[10] to subdivide our subset of primary nodal DLBCL into GCB and non-GCB (**Figure 1**).

Statistical procedures. Analysis of data was carried out by IBM computer using the Statistical Program for Social Science version 15 (SPSS Inc., Chicago, IL., USA). Description of quantitative variables were expressed as mean, standard deviation, and range, and description of qualitative variables were expressed as number and percent. Chi-square test (χ^2) and Fisher exact test (was performed in tables containing value less than 5) was used to compare between groups A and B, regarding the presence of B symptoms, PS scale, affected extranodal sites number, disease stage, high LDH level, IPI score, and patients outcome. Student t-test was used to assess the statistical significance of the difference between groups A and B regarding the mean value of age. Overall survival (OS) analysis was performed at the univariate level by means of Kaplan-Meier techniques, Log-rank test was used to calculate *p*-value.[19] All variables were individually evaluated in a hazard ratio model. Variables significantly related to OS were then included in the multivariate Cox proportional hazard regression model. Results of the regression analyses were expressed as a hazard ratio (HR) with its 95% confidence interval (CI). A *p*-value of <0.05 was considered statistically significant, while *p*-value ≤0.001 was considered statistically highly significant. The end-point of the study was OS, which was calculated from the date of diagnosis until last follow-up or death.

Results. The results of this study are illustrated in **Tables 1-3**, and **Figures 2 & 3**. The studied patients include 19 men and 11 women with a men to women ratio of 1.7:1. Their age ranged from 22-77 (mean: 49.2 ± 13.0 years), with a follow up period not less than 24 months for surviving patients. Applying the IHC algorithm described by Hans et al,[10] patients was classified as GCB (group A), and non-GCB (group B). Group A included 10 men and 7 women with men:women ratio of 1.4:1. Their ages ranged from 28-60 years. Group B include

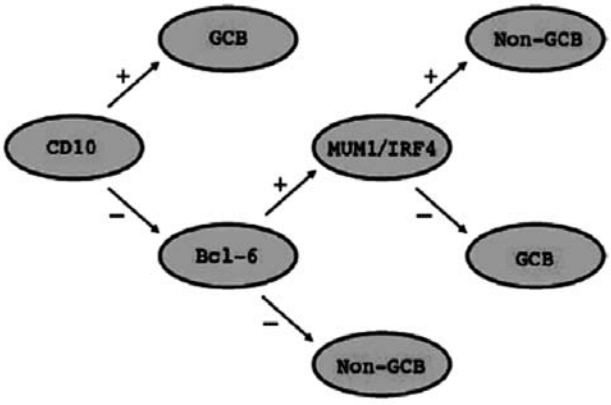

Figure 1 - The immunohistochemistry (IHC) algorithm for assigning cases to GCB and non-GCB subgroups based on immunohistochemistry profile.[10] CD: cluster of differentiation; GCB: germinal center B-cell-like; BCL6: B-cell/lymphoma 6; MUM1/IRF: multiple myeloma-1/interferon regulatory factor-4.

Table 1 - Comparison between immunohistochemically defined GCB and non-GCB DLBCL phenotypes regarding clinical, laboratory characteristics, and patients' outcome.

Variables	Group A n=17	Group B n=13	*P*-value
Age, mean ± SD	47.9 ± 8.9	50.8 ± 17.3	0.5
	n (%)		
B symptoms			0.4
Yes	8 (47)	8 (61)	
No	9 (53)	5 (39)	
Performance status			0.0001
1	16 (94)	4 (31)	
>1	1 (6)	9 (69)	
Extranodal sites			0.07
<2	17 (100)	10 (77)	
≥2	0	3 (23)	
Staging			0.72
I/II	8 (47)	5 (39)	
III/IV	9 (53)	8 (61)	
Lactate Hydrogenase			0.01
Normal	7 (41)	0 (0)	
High	10 (59)	13 (100)	
IPI risk			0.16
Low/Low intermediate	10 (59)	4 (31)	
Intermediate high/High	7 (41)	9 (69)	
Outcome			0.008
Good	14 (82)	4 (31)	
Bad	3 (18)	9 (69)	

GCB - germinal center B-cell like, DLBCL - diffuse large B-cell lymphoma, IPI - international prognostic index

Table 2 - Predictive value of clinical, laboratory features, and DLBCL phenotypes on OS.

Variables	Means for survival time (months)		*P*-value
	Estimate	Standard error	
GCB antigen expression			0.002
Group A	46.9	5.8	
Group B	19.6	5.4	
IPI risk			0.08
Low/Low intermediate	48.37	4.92	
Intermediate high/High	26.20	6.22	
Lactate Hydrogenase			0.02
Normal	-	-	
High	20.0	1.05	
Performance status			0.01
1	44.4	5.4	
2 & 3	18.6	6.6	
Staging			0.40
I/II	21.2	1.2	
III/IV	30.9	6.6	

DLBCL - diffuse large B-cell lymphoma, OS - overall survival, GCB - germinal center B-cell like, IPI - international prognostic index

Table 3 - Multivariate Cox proportional hazard regression for mortality risk.

Variables	Number of deaths/total (%)	Unadjusted HR (95% CI)	Adjusted HR (95% CI)	*P*-value
Phenotype				0.008
Group A	3/17 (18)	3.30	6.07	
Group B	9/13 (69)	(0.52-21.10)	(1.6-22.9)	
LDH				0.99
Normal	0/7	35.35	∞	
High	12/23 (52)	(0.18-6816.11)	(0-∞)	
PS				0.98
1	5/20 (25)	3.96	1.02	
>1	7/10 (70)	(1.25-12.54)	(0.20-5.2)	

LDH - lactate hydrogenase, PS - performance status, CI - confidence interval, HR - hazard ratio, ∞ - infinity

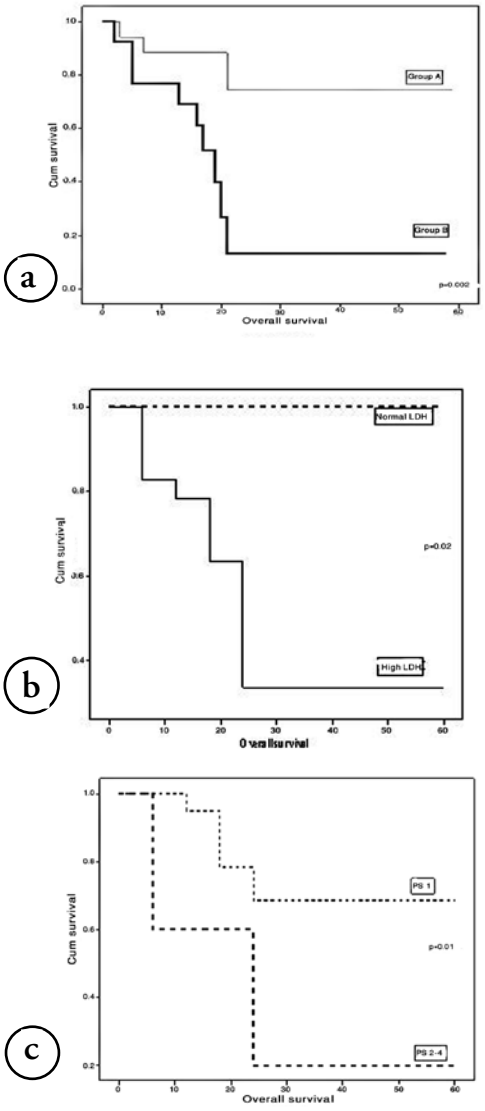

Figure 2 - Kaplan Meier curves illustrating overall survival of the patients **a**) germinal center B-cell like (GCB), **b**) lactate dehydrogenase (LDH) level, **c**) performance status (PS) score.

9 men and 4 women with men:women ratio of 2.2:1. Their age ranged from 22-77 years.

Association between DLBCL phenotypes and clinical and laboratory data (Table 1). Both high LDH level and PS >1 were statistically significantly associated with postgerminal center antigen expression. A statistically insignificant difference between group A and B regarding the presence of B symptoms, number of extranodal sites involvement, Ann Arbor staging system, and IPI score was found (p>0.05).

Prognostic value of DLBCL phenotypes in patients' outcome (Table 2). There was a statistically significant association between GCB antigen expression and outcome, where 9 of group B patients had bad outcome (2 progressive disease, 2 relapsed, and 5 died), only 3 of group A patients died (p=0.008).

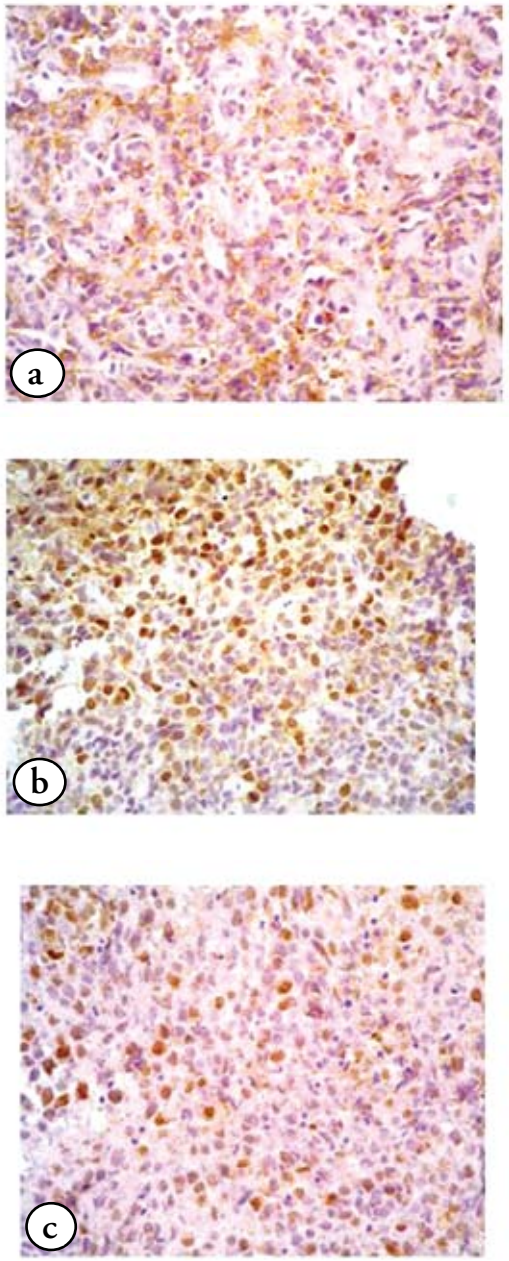

Figure 3 - Immunohistochemical staining (400x) for **a**) positive staining of cluster of differentiation 10, **b**) positive staining of B-cell/lymphoma 6, and **c**) positive staining of multiple myeloma-1/interferon regulatory factor-4.

Table 4 - Patients' characteristics in different studies.

Studies	N	Methods*	Treatment	GCB group (%)
Current study	30	IHC	CHOP & CHOP like regimen	(57)
Hans et al[10]	152	IHC (TMA)	Anthracycline-based regimens	(42)
Berglund et al[11]	161	IHC	CHOP & CHOP like regimen	(52)
Poulsen et al[21†]	52	DNA microarray	Anthracycline-based regimens ± RT	(40)
Shia et al[22]	54	IHC & PCR for BCl2 translocation	-	(50)
Liu et al[23†]	163	IHC (TMA)	CHOP & CHOP like regimen	(29)
Oh and Park[24]	51	IHC (TMA)	-	(36)

*methods used for classification of the patients into germinal center-B-cell like (GCB) and non-GCB, †both studies had classified the patients into 3 groups. IHC - immunohistochemistry, TMA - tissue microarray, DNA - deoxyribonucleic acid, PCR - polymerase chain reaction, BCl2 - B-cell/lymphoma 2, CHOP - cyclophosphamide, doxorubicin, vincristine, and prednisone, RT - radiation therapy

Table 5 - Antibodies used for immunohistochemical stains in different studies.

Studies	CD10	BCL6	MUM1/IRF4
	Source (cutoff value for staining positivity [%])		
Current study	Lab Vision, Fremont, USA (30)	Lab Vision, Fremont, USA (30)	DAKO, Glostrup, Denmark (30)
Hans et al[10]	Ventana, Pittsburgh, USA (30)	Santa Cruz, California, USA (30)	Santa Cruz, California, USA (30)
Berglund et al[11]	Ventana, Pittsburgh, USA (30)	DAKO, Denmark (30)	Santa Cruz, California, USA (30)
Poulsen et al[21]	DAKO, Glostrup, Denmark (30)	DAKO, Denmark (30)	DAKO, Glostrup, Denmark (30)
Shia et al[22]	Novocastra, Newcastle upon Tyne, UK (75)	DAKO, Glostrup, Denmark (10)	Not carried out
Liu et al[23]	Novocastra, Newcastle upon Tyne, UK (30)	DAKO, Glostrup, Denmark (30)	DAKO, Glostrup, Denmark (30)
Oh and Park[24]	Ventana, Pittsburgh, USA (30)	Ventana, Pittsburgh, USA (30)	DAKO, Glostrup, Denmark (30)

In the above studies, immunohistochemistry staining was performed according to a standard 3 step immunoperoxidase method. CD - cluster of differentiation, BCL6 - B-cell/lymphoma 6, MUM1/IRF4 - multiple myeloma-1/interferon regulatory factor-4

Overall survival and multivariate analysis (Tables 3 & 4, Figure 2). Based on Kaplan Meier survival curve, non-GCB phenotype (p=0.002), high LDH level (p=0.02), and PS >1 (p=0.01) were all associated with increased mortality risk. The median OS time was 46.9 months in group A patients compared with 19.6 months in group B patients (HR=3.30; 95% CI=0.52-21.10). Using multivariate Cox proportional hazard regression analysis, non-GCB phenotype was found to be the most predicting factor. The risk of mortality was nearly 6 folds in group B compared with group A (HR=6.07; 95% CI=1.6-22.9; p=0.008).

Discussion. The DLBCLs are a heterogeneous group of malignancies with different clinical presentations and courses. Numerous biological prognostic factors have been analyzed with an attempt to improve the subdivision of the disease, but such effort has failed because of conflicting results.[20] In recent years, knowledge of DLBCL has increased dramatically in light of the repeated finding of a GCB and a non-GCB group.[11] The aim of the present work is to study the expression of GCB/ABC-related proteins to get optimal stratification of DLBCL patients, and correlate this with the established clinical parameters. In this study, 57% of the studied patients were grouped as GCB phenotype. This matches previous reports by other studies.[10,11,21,22] However, Liu et al[23] and Oh and Park[24] recorded lower values (**Table 4**). This may be explained by different sources of antibodies used. Variable cut off values for positive staining may also influence the results. Although, 10% cutoff level is the most commonly employed, yet several researchers[10,11] reported that this level might be too low to subdivide DLBCL into manageable subgroups (**Table 5**).

In the current study, group A and B patients were comparable regarding disease stage, presence of B symptoms, IPI score, and extranodal sites involvement, and this matches other studies (**Table 6**). We found that serum LDH level and PS were significantly higher among non-GCB group, but this was not reported in other studies (**Table 6**). Similar to previously published reports, this study demonstrated that GCB group showed a significantly better OS than the non-GCB group (**Table 6**).[10,11,23] Moreover, using multivariate analysis GCB antigen expression was the most predicting factor of outcome. This is in line with the studies by Berglund et al,[11] and Liu et al.[23] Nearly all studies of prognostic indicators in DLBCL were based on clinical outcome following treatment with anthracycline-containing chemotherapy regimens. However, new strategies such as the addition of immunotherapy in the form of rituximab (R) to combination chemotherapy may be associated with different biologic or clinical prognostic factors.[14]

In conclusion, patients with GCB phenotype had a significantly better outcome and survival, compared to patients with the non-GCB phenotype using the 3-marker model expression (CD10, BCL6, and MUM1/IRF4).

The current study has several limitations as the addition of rituximab to chemotherapy, which is now the standard of care in the treatment of DLBCL was not adopted. Also at the time of this study, FDG-PET scanning was still not included in the response definitions. It is recommended that this immunohistochemical staining be further evaluated prospectively on a larger number of patients for possible incorporation in the routine evaluation of all new DLBCL cases. This might be the first step towards a deeper understanding of the biology of the heterogeneous group of DLBCL. Moreover, these immunohistochemical prognostic factors have to be re-evaluated in the post-R era to obtain additional tools for tailoring treatment in this group of heterogeneous patients.

Table 6 - Comparison between GCB and non-GCB phenotypes regarding clinical, laboratory characteristics, and survival in different studies.

Parameters	Current study	Hans et al[10]	Berglund et al[11]	Poulsen et al[21]	Lui et al[23]	Oh & Park[24]
Age	NS	NS	-	-	NS	NS
B symptoms	NS	-	NS	-	-	-
PS	S	NS*	-	-	NS	NS*
Extranodal sites ≥2	NS	NS	-	-	S	NS
Ann Arbor staging	NS	NS	NS	-	NS	NS
LDH (normal versus high)	S	NS	-	-	NS	NS
IPI	NS	NS	NS	-	NS	NS
OS	S	S	S	S	S	NS
PFS	-	S	S	-	S	-

*Karnofsky score. GCB - germinal center B-cell-like, PS - performance status, LDH - lactate hydrogenase, IPI - international prognostic index, OS - overall survival, PFS - progression free survival, S - statistically significant, NS - non significant.

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