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ORIGINAL ARTICLE

Characterisation of immunogenotypes of diffuse large B-cell lymphoma

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Abstract

Introduction: Diffuse large B-cell lymphoma (DLBCL) is the most common aggressive type of non-Hodgkin lymphoma with variable clinical outcomes. The immunogenotypic features of this heterogeneous disease in Malaysia were not well characterized. *Materials & Methods:* In total 141 local series of DLBCL cases from UKM Medical Centre were retrospectively studied. *Results:* Of these cases, we classified our patients into two subtypes: 32.7% (37/113) GCB and non-GCB 67.3% (76/113) by Hans algorithm and the results showed strong agreement with the results by Choi algorithm ($\kappa = 0.828$, $P<0.001$). Survival analysis indicated significant difference in between GCB and non-GCB subtypes (P=0.01), elevated serum LDH (P=0.016), age more than 60-year-old (P=0.021) and the presence of B symptoms (P=0.04). We observed 12% DLBCL cases were CD5 positive and 81.8% of them died of the disease (P=0.076). Analysis on the dual expression of MYC/BCL2 revealed that there is no significant difference in DE and non-DE groups (P=0.916). FISH study reported there were 9.22% (13/141) rearranged cases observed in our population at which highest frequency of *BCL6* gene rearrangement (76.9%), followed by *MYC* (15.4%) and *BCL2* (7.7%); no *BCL10* and *MALT-1* gene rearrangement found regardless of using TMAs or whole tissue samples. More cases of MYC protein overexpression observed compared to MYC translocation. *Conclusion:* Relatively lower frequency of GCB tumours and low gene rearrangement rates were observed in Malaysian population. A national study is therefore warranted to know better the immunogenotypic characteristics of DLBCL in Malaysia and their implications on the survival.

Keywords: diffuse large B-cell lymphoma, immunohistochemistry, fluorescent in situ hybridization, gene rearrangement, Malaysia

INTRODUCTION

Diffuse large B-cell lymphoma is a clinically and biologically heterogeneous B-cell lymphoma with different subtypes in accordance with the gene expression profiling. The highly aggressive nature of this lymphoid malignancy requires proper diagnosis and early detection. According to Malaysian National Cancer Registry Report from 2007 to 2011 reported by National Cancer Institute, in ten most common cancers among Malaysian, lymphoma was ranked at fourth with 5.2% of all cancers diagnosed during that period [1] with significant number of patients died of the disease though it is curable with the available immunochemotherapy such as RCHOP treatment (combination of rituximab with cyclophosphamide, doxorubicinm vincristine and prednisolone). Currently available prognostic tools might require to be improved for better risk-stratification of patients and efficient planning of targeted therapy strategy specifically among the Malaysian population.

The gold standard in the diagnosis of diffuse large B-cell lymphoma is a histopathological assessment using immunohistochemical methods. The diagnostic immunohistochemical markers for DLBCL include CD19, CD20, CD22, CD75 CD79a, PAX5, CD30, IgM>IgG>IgA, CD10, MUM1, BCL2, BCL6, Ki67, CD5, OCT-2, BOB1, GCET1, c-MYC, FOXP1 and LMO2.[2-6]

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This lymphoma is divided into two prognostic groups namely germinal-centre B-cell subtype (GCB) and non-germinal centre B-cell subtype (non-GCB) by gene expression analysis (GEP), based on the cell of origin. However, it is impractical to perform GEP on every single case due to the costly expenses, therefore various algorithms were developed to predict the cell of origin as well as the survival. Both Hans and Choi algorithms are well-established immunohistochemical algorithms widely used to identify these subtypes which were reported pragmatically useful to predict the prognosis of patients with DLBCL. In 2004, Hans et. al reported that the expression of CD10, BCL6, MUM1, BCL2 and cyclin D2 are useful predictors of survival similar to the cDNA microarray in which the combination of CD10, BCL6 and MUM1 can divide DLBCL into GCB and non-GCB subtypes.[7] Choi and co-workers developed a new improved immunostain algorithm using GCET1, CD10, BCL6, MUM1 and FOXP1 with 93% concordance with the gene expression profiling (GEP) classification of diffuse large B-cell lymphoma (~80% by Hans algorithm). This algorithm is reported significantly more accurate than Hans algorithm.[3]

It has been suggested that genetic and molecular features may modify disease aggressiveness and treatment response in DLBCL. Of interest are gene rearrangements (such as those involving *BCL2* and *BCL6*) and protein expression (such as BCL2 and BCL6) that have been shown to have major prognostic values.

Diffuse large B-cell lymphoma carries heterogenous chromosomal abnormalities.[8-12] These include t(14;18) (q32;q21) involving the *BCL-2* gene and 3q27 for *BCL-6* gene which accounts for 20% to 30% of DLBCL cases. Furthermore, the *MYC* gene that is located on 8q24, is rearranged in 5% to 15% of cases. The *MALT-1* gene on 18q21 which lies about 5Mb centrometric to *BCL-2*, has been reported by Sanchez-Izquierdo *et al* as translocated and amplified in DLBCL, but this occurrence needs to be evaluated.

The main aim of the present study was to investigate the degree of agreement of both Hans and Choi algorithms as well as their correlation to survival rate. Besides, we also evaluated the importance of CD5-positive diffuse large B-cell lymphoma as well as BCL2/MYC dual expressor lymphoma (DEL) which were reported in Western to have an adverse impact of the prognosis. Besides immunophenotypic profiles, the next aim was to investigate a local series of DLBCLs to detect *BCL2*, *BCL6*, *MYC*, *BCL10* and *MALT-1* gene rearrangements using fluorescent *in situ* hybridization (FISH) techniques. The results of the molecular studies were correlated with immunophenotypic profiles and patients' clinical data to understand the clinical significance of the genetic abnormalities. Knowledge of the immunohistochemical features and the lymphoma-associated chromosomal translocations may contribute to better patient stratification and a more tailored therapeutic approach in Malaysia.

MATERIALS AND METHODS

Study design:

This retrospective study recruited one hundred and forty-one (141) cases of diffuse large B-cell lymphoma in the form of formalin-fixed paraffin embedded tissue blocks from the Department of Pathology, UKM Medical Centre. The patients were diagnosed between 2004 and 2010 and they had no history of other lymphoproliferative disorders. The study was approved by the Ethics Committee of UKM, Faculty of Medicine (DIP-2012-10/3). Clinical data such as sites of specimens, subtypes, age, gender, race, revised IPI and treatments, survival status and performance status were retrieved.

Construction of Tissue Microarray (TMA):

The immunohistochemical staining and FISH test were performed on the tissue microarray (TMA) sections in Histopathology Unit, Department of Pathology, PPUKM. The haematoxylin and eosin (H&E) slides were reviewed by a pathologist to select the representative areas of tumour tissues before preparing the cores using the punch arrayer (Alphelys MTA Booster, Plaisir, France). An Excel file was created with the details of donor blocks, for instance patient ID, block ID and tissue types. Besides, the TMA maps were prepared prior to the other procedures to facilitate the array coordinates.

The instrument created holes in a recipient paraffin block with defined array coordinates. Two representative areas of 0.6mm in diameter tissue core were taken from each tumour (donor) block and arrayed into recipient paraffin blocks of 20x20 mm, with 1.4 mm spacing between the cores, creating a maximum of 8 x 7 dots in the different blocks consisting of 56 cores in a single recipient block. Three orientation cores were placed on the top of the array for

TABLE 1: List of antibodies and staining conditions

No	Antibody	Dilution	Source	Clone
1	BCL2 (mouse monoclonal, IgG1)	1:100	Dako, Denmark	124
2	BCL6 (mouse monoclonal, IgG1)	1:100	Dako, Denmark	PG-B6p
3	CD10 (mouse monoclonal, IgG1)	Ready-to-use	Dako, Denmark	56C6
4	CD20 (mouse monoclonal, IgG2a)	1:200	Dako, Denmark	L26
5	CD79a (mouse monoclonal, IgG1)	Ready-to-use	Dako, Denmark	HM57
6	MUM1 (mouse monoclonal, IgG1)	Ready-to-use	Dako, Denmark	MUM1p
7	CD5	Ready-to-use	Dako, Denmark	JCB117
8	GCET1	Neat	Abcam Inc., Cambridge	RAM341B/C1/C2
9	FOXP1	1:80	Abcam Inc., Cambridge	JC12
10	MYC	Ready-to-use	Epitomics	Y69

orientation identification purpose. The positive and negative control tissues were included to serve as orientation cores as well as to validate the results.

Immunohistochemistry:
Immunohistochemical studies were performed on paraffin sections of 4 micrometre (μm) thickness, using the modified conventional antigen retrieval protocol [13] and the chromogen was diaminobenzidine. Suitable negative and positive controls were used along with the staining for each antibody. List of antibodies used for immunohistochemistry and staining conditions is shown in Table 1.

CD10, BCL6 and MUM1 staining were considered positive if more than 30% of neoplastic cells were scored positive. For insufficient tissues and artefact observed in TMAs, the results were reviewed using whole tissue sections. The stained slides were interpreted by two experienced haematopathologists (NM and NHH) under light microscopes, who were blinded for to other stains as well as follow up data, while any discrepancy was resolved by consensus review.

Fluorescent *in situ* hybridization (FISH):
3-4 μm thick of paraffin tissue sections were prepared and mounted on charged slides (X-tra® Slides, Leica Biosystems, Nussloch, Germany). The histology FISH accessory kit (Dako, Glostrup, Denmark) was used to together with FISH probes on the tissue sections to produce optimally satisfied results. Table 2 shows the conditions utilized during the process of preparing FISH slides. Five ready-to-use dual-colour break apart probes (*BCL2, BCL6, BCL10, MUM1* and *MALT-1* by Dako, Glostrup, Denmark) were used to perform interphase FISH for all cases. Validation of TMAs' results was done using whole tissue section to confirm the results produced by TMAs. The fluorescence microscope with DAPI, Texas Red and FITC double or single filters (Olympus) was used for the interpretation of the results.

Statistical Analysis:
In this study, demographic data was presented as frequency counts with percentage for categorical variables. Age was reported as median with range as well as below and above 60 years old whereas gender was reported as ratio. Pearson's Chi-square test or Fisher's exact test was performed to assess whether the frequency of a condition is significantly different between two or more groups. Pearson's was selected if none of the cells have expected count less than 5 and the study recruited more than 40 samples whereas

TABLE 2: **Reagents and temperatures for fluorescent in situ hybridization (FISH)**

No	Reagent	Temperature (°C)
1	Diluted Pre-Treatment Solution	95-99
2	Pepsin	2-8
3	Diluted Wash Buffer	20-25
4	Diluted Stringency Buffer A	20-25
5	Diluted Stringency Buffer B	65 (±2)
6	Fluorescence Mounting Medium	2-25
7	Coverslip Sealant	20-25

Fisher's was used in the condition of more than 20% of cells have expected count less than 5. Logistic regression was used to analyze the data with dichotomous outcome, regardless type of the type of independent variables as well as to measure the odd ratio. Kaplan-Meir method was used to examine the survival probability and log-rank test was conducted to make a comparison of the difference between the two groups in which P-value of <0.05 was statistically significant. The prognostic value of the potential factors affecting overall survival was evaluated by multivariate analysis using the Cox proportional hazards model. Overall survival (OS) refers to the interval from the date of diagnosis until death from any cause, or for the survivors, it is defined as the time from diagnosis to the last contact. SPSS 20.0 software package (Chicago, IL, USA) was used to perform the statistical analysis throughout this study.

RESULTS

Patient demographic and clinical data (Table 3):

a. Age and genders

There were in total of 141 patients included in this study where 79 (56%) were males and 62 (44%) were females. The ratio of female to male was 1:1.27. There were 53 (37.6%) patients below 60 years old and 88 (62.4%) patients with age equal or older than 60 years old. Their age is ranged from 5 to 84 years with a peak incidence between 60 to 75 years old. The mean of the age is 60.16 years.

b. Race

In our study cohort, there were 77 (54.6%) Malays, 55 (39.0%) Chinese, 2 (1.4%) Indians and 7 (5.0%) of other races.

c. Haemoglobin level

Haemoglobin (Hb) level was determined at the initial diagnosis to assess if the patients have anaemia. The normal Hb level for male is more than 13.5 g/dl and female more than 11.5 g/dl. There is no severe anaemia for all the male patients, with majority (43, 61.4%) distributed in mild anaemic range and 27 (38.6%) of the male patients had Hb level above 13.5 g/dl which is within a normal range. There was only one (1.7%) female patient showed severe anaemia as the Hb level was below 6 g/dl and most of them (36, 62.1%) had moderate anaemic conditions. There were 21 (36.2%) female patients exhibited normal Hb level which more than 11.5 g/dl.

d. Serum lactate dehydrogenase (LDH) Level

The data of LDH levels were retrieved from 119 patients in which the LDH level ranged from 261 to. 20000 unit/litre (u/l). There were 28 (23.5%) patients with normal LDH level (≤ 423 u/l), 51 (42.9%) presented with LDH level between 424 – 1000 u/l and 40 (33.6%) had LDH level more than 1000 u/l.

e. Ann Arbor Stage

Most of our patients were diagnosed at the late stage in which 52 (46.4%) patients at stage IV disease, 29 (25.9%) at stage III whereas 16 (14.3%) patients at stage I and 15 (13.4%) patients at stage II disease.

f. Revised International Prognostic Index (R-IPI)

The R-IPI was calculated using online R-IPI calculator (Website: https://qxmd.com/calculate/calculator_64/diffuse-large-b-cell-lymphoma-prognosis-r-ipi), using five risk factors namely age, LDH level, extranodal sites, Ann Arbor Stage and ECOG performance status. The

TABLE 3: Demographics and clinical presentation of patients with DLBCL in UKM Medical Centre

Characteristics	All patients		GCB	Non-GCB	*P**	Odd Ratio	95% CI for OR	
	N	Percentage (%)					*Lower*	*Upper*
Age in years								
<60	53	37.6	20	25				
≥ 60	88	62.4	17	51	*0.033*	2.400	1.074	5.365
Youngest (Min)	5							
Eldest (Max)	84							
Mean	60.1							
Gender								
Female	62	44	16	34	0.881	1.062	0.481	2.346
Male	79	56	21	42				
Race								
Malay	77	54.6	20	41	0.121			
Chinese	55	39.0	11	34	0.352	1.508	0.635	3.580
India	2	1.4	1	0	1.000	0.000	0.000	-
Others	7	5.0	5	1	*0.039*	0.098	0.011	0.892
Haemaglobin level						NA	NA	NA
Male								
≤ 6 g/dl	0	0	0	0				
6.1 – 13.5 g/dl	43	61.4	12	24	0.628			
> 13.5g/dl	27	38.6	6	16				
Female								
≤ 6 g/dl	1	1.7	1	0				
6.1 – 11.5 g/dl	36	62.1	9	19	0.375			
> 11.5g/dl	21	36.2	6	11				
LDH level								
≤423 u/l	28	23.5	5	20	0.202			
424 – 1000 u/l	51	42.9	17	24	0.079	0.353	0.111	1.126
>1000 u/l	40	33.6	9	20	0.359	0.556	0.158	1.952
Ann Arbor stage								
I	16	14.3	2	10	0.257			
II	15	13.4	5	10	0.334	0.400	0.062	2.568
III	29	25.9	10	12	0.107	0.240	0.042	1.360
IV	52	46.4	10	31	0.576	0.620	0.116	3.317
R-IPI								
Very Good	12	14.8	6	5	0.121			
Good	19	23.5	5	14	0.129	3.360	0.702	16.080
Poor	50	61.7	10	35	*0.041*	4.200	1.057	16.683
Performance Status (ECOG)								
0	24	35.3	7	14	0.209			
1	28	41.2	2	17	0.100	4.250	0.759	23.813
2	5	7.4	2	2	0.529	0.500	0.058	4.335
3	10	14.7	4	3	0.272	0.375	0.065	2.159
4	1	1.5	0	1	-	-	-	-
Bone Marrow Involvement								
Yes	16	17.2	5	7				
No	77	82.8	19	47	0.378	0.566	0.160	2.006

Characteristics	All patients		GCB	Non-GCB	*P**	Odd Ratio	95% CI for OR	
	N	Percentage (%)					*Lower*	*Upper*
B Symptoms								
Present	38	30.9	14	16	*0.034*	0.381	0.156	0.931
Absent	85	69.1	18	54				
No. of nodal sites (nodal disease)								
0	39	32.2	6	23				
Present extranodal	82	67.8	26	43	0.107	0.431	0.155	1.199

* GCB versus non-GCB. *Italics* indicate statistically significant values. N = number. R-IPI - Revised International Prognostic Index

patients were assigned into three prognostic groups: Good, Intermediate and Poor[14]. Our study adopted revised IPI (R-IPI) in the era of rituximab in which the score can differentiate patients into three groups: very good, good and poor. Out of 81 patients with complete data, there were 12 (14.8%) patients with very good prognosis, 19 (23.5%) with good prognosis and 50 (61.7%) of them with poor prognosis.

g. Performance Status by the Eastern Cooperative Oncology Group (ECOG)

Information regarding performance status (ECOG score) was available in 68 patients. Most of the patients (52, 76.5%) presented score 0 – 1, 5 (7.4%) patients with score 2, 10 (14.7%) patients score 3 and only 1 (1.5%) patient had score of 5.

h. Bone marrow involvement

A total of 16 (17.2%) patients showed the bone marrow involvement at diagnosis while 77 (82.8%) patients did not have bone marrow involvement.

i. B symptoms

Information on B symptoms (for instance lose weight, night sweat, and fever) was available for 123 patients where 38 (30.9%) patients presented with symptoms while 85 patients had no B symptoms.

j. Site of tumour and number of nodal sites involved

Out of the 141 cases of DLBCL, 82 cases presented as extranodal sites. Twenty-eight (34.1%) patients showed the presence of one nodal site, 19 (23.2%) patients had two nodal sites, 8 (9.8%) of them exhibited three nodal sites, 26 (31.7%) patients had 4 nodal sites and 1 (1.2%) patient showed five nodal sites.

Statistical Analysis of Demographic Data

By using Pearson's Chi square test and further analysed by logistic regression method, between GCB and non-GCB tumours, there were statistical significance in age ≥ 60 (P = 0.033; OR = 2.400, 95% CI 1.074, 5.365); races (P = 0.039; OR = 0.098, 95% CI 0.011, 0.892); revised International Prognostic Index – poor (P = 0.041; OR = 4.200, 95% CI 1.057, 16.683); presence of B symptoms (P = 0.034; OR = 0.381, 95% CI 0.156, 0.931).

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Immunophenotypic profiles

Immunophenotypic profile of all our samples yielded positivity rates for CD10 (29/110, 26.4%), GCET1 (22/106, 20.8%), BCL6 (37/113, 32.7%), MUM1 (73/111, 65.8%), FOXP1 (77/104, 74.0%), CD20 (102/112, 91.1%), CD5 (11/92, 12%), MYC (29/70, 41.4%) and BCL2 (31/67, 46.3%). Fig. 1 and Fig. 2 show the immunohistochemical staining of tumour cells in this study.

Using Han's algorithm to determine subtypes (GCB and non-GCB), we found IHC stains that are statistically significant include CD10 (P < 0.01), BCL6 (P < 0.01), MUM1 (P = 0.02) which are the key markers used in this algorithm for subtype classification; CD10 with Pearson coefficient, r = -0.84 exhibited relatively strong relationship with those subtypes. These results are summarized in Table 4.

Concordance of Gene Expression Profiling-Related Immunohistochemical Algorithms

In this study, both well-established immunohistochemical algorithms namely Hans and Choi were used to divide the cases into GCB and non-GCB subtypes. There were 113 cases and 110 cases suitable for subgrouping by Hans

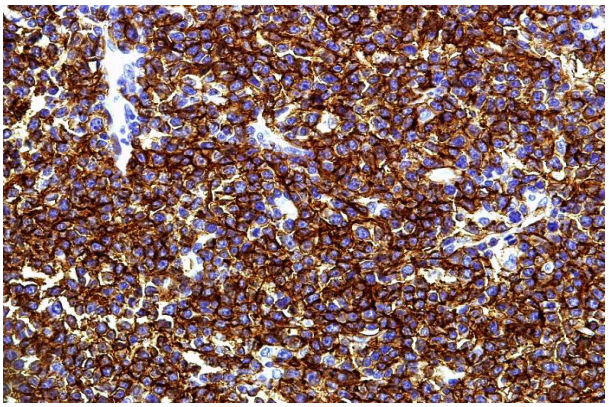

FIG. 1: The neoplastic cells of diffuse large B-cell lymphoma diffusely express CD20 which is also one of the reliable pan B-cell markers (X400).

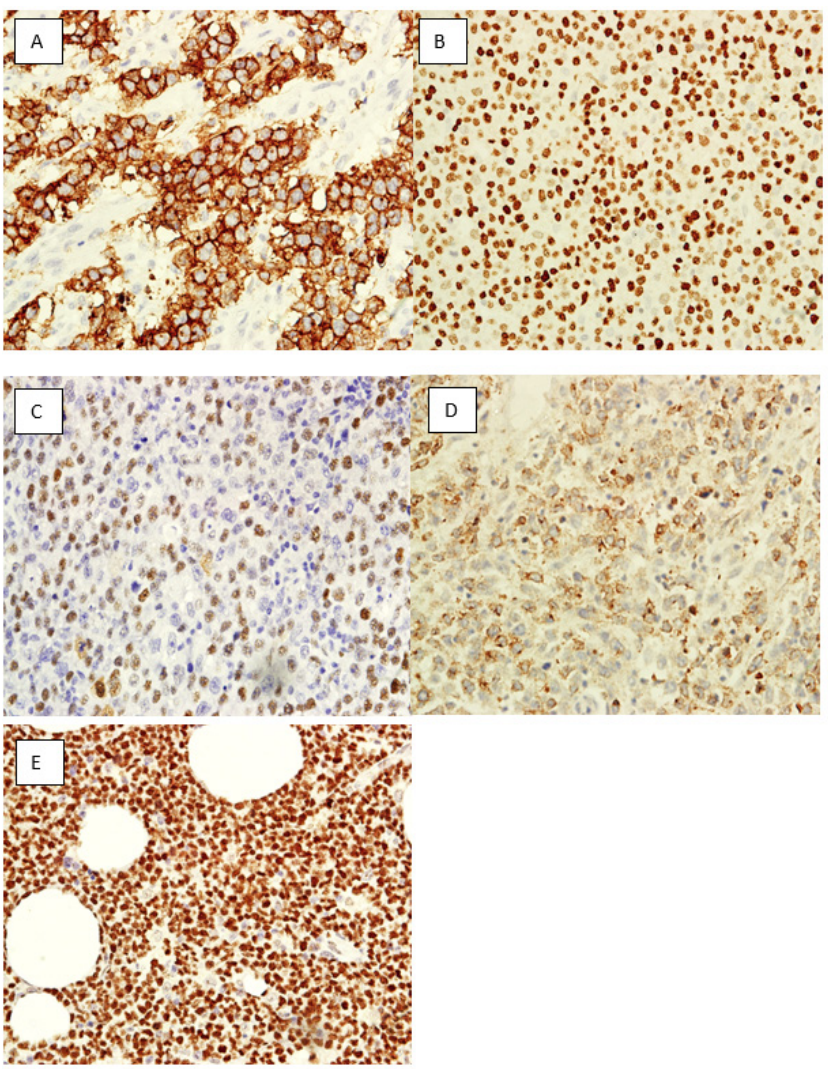

FIG. 2: Immunohistochemical staining of tumour cells at magnification power of 400X for cell-of-origin study by Hans and Choi algorithms. A: CD10 positive on cell membrane, B: MUM1 positive in cell nucleus, C: BCL6 positive in cell nucleus, D: GCET1 positive on cell membrane and E: FOXP1 positive in cell nucleus.

TABLE 4: Immunohistochemical staining on DLBCL samples

IHC marker	Total (N)	Percentage (%)	GCB (Han's algorithm)	Non-GCB (Han's algorithm)	*P**
CD10					
Positive	29	26.4	29	0	*<0.01*
Negative	81	73.6	8	73	r = -0.84
GCET1					
Positive	22	20.8	7	15	
Negative	84	79.2	32	52	0.667
BCL6					
Positive	37	32.7	20	17	*<0.01*
Negative	76	67.3	16	59	r = -0.33
MUM1					
Positive	73	65.8	15	57	*0.02*
Negative	38	34.2	19	19	r = 0.300
FOXP1					
Positive	77	74.0	23	53	0.181
Negative	27	26.0	12	15	
CD20					
Positive	102	91.1	33	63	0.950
Negative	10	8.9	3	6	
CD5					
Positive	11	12.0	1	10	0.079
Negative	81	88.0	26	47	
MYC					
Positive	29	41.4	10	19	0.797
Negative	41	58.6	15	25	
BCL2					
Positive	31	46.3	11	19	0.522
Negative	36	53.7	16	20	

*GCB versus non-GCB

and Choi respectively, missing cases were those with insufficient samples. As for Hans algorithm, 37/113 (32.7%) were GCB subtypes and 76/113 (67.3%) were classified as non-GCB. Meanwhile, Choi algorithm produced 37/110 (33.6%) of GCB subtypes and 73/110 (66.4%) of non-GCB subtypes. The results are tabulated in Table 5.

Cohen's kappa analysis was conducted to determine if there was an agreement between the two algorithms as our data are paired observation of the same phenomenon, meaning that both algorithms are measuring the same phenomenon ie.: GCB and non-GCB subtypes.

From the crosstabulation table produced from SPSS, it is noted that 31 patients displayed GCB subtypes and 65 patients showed non-GCB subtype as shown by both algorithms. In addition, there were in total of 8 patients (i.e., 5+3) for whom the algorithms showed discrepancy (Table 6).

The Symmetric measures table showed the Cohen's kappa (κ) (range: -1 to 1) which is a statistic to present *chance agreement*. The κ value of our data is 0.828, meaning that the proportion of agreement is *over and above* chance agreement. In other words, there was

TABLE 5: Classification of DLBCL into GCB and non-GCB subtypes using Hans and Choi algorithms

	GCB, n (%)	Non-GCB, n (%)
Hans algorithm (113 cases)	37 (32.7)	76 (67.3)
Choi algorithm (110 cases)	37 (33.6)	73 (66.4)

TABLE 6: Crosstabulation of Hans and Choi algorithms on the subtypes of DLBCL

		Choi algorithm		Total
		GCB	NON-GCB	
Hans algorithm	GCB	31	3	34
	NON-GCB	5	65	70
Total		36	68	104

strong agreement or high concordance of the two algorithms, $\kappa = 0.828$ (95% CI; $p<0.005$) (Table 7), our kappa coefficient is statistically significant.[15,16]

Survival analysis in subtypes of diffuse large B-cell lymphoma

We found that the survival experience is significantly distinguished ($P = 0.001$) between these two studied subtypes by the mean of log-rank test. Kaplan-Meier curve showed that patients with GCB subtypes have better survival outcome in comparison to the patients with non-GCB subtype (Figure 3). The overall 2-year and 4-year survival rates for GCB patients are 60.6% and 48.5% respectively whereas the survival rates for non-GCB patients are 23.0% and 10.5% respectively. The median survival time (the time at which 50% patients have died) are 40 months (standard error= 16.7) for GCB patients and 14 months (standard error= 1.1) for non-GCB. Cox regression analysis (Table 8) revealed that patients who have non-GCB are 129% more likely to die compared to those who have GCB subtypes (HR = 2.29, 95% CI, 1.4 – 3.75; $P<0.01$).

Survival Analysis of the Presence of B Symptoms in Diffuse Large B-cell Lymphoma

B symptoms are collectively general symptoms associated with the rapidly developed lymphoma and they are always identified during the staging

TABLE 7: Symmetric Measures

		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement	Kappa	0.828	0.058	8.450	.000
N of Valid Cases		104			

TABLE 8: Cox Regression Test (Subtypes)

Subtype[1]	N	No. of Death	Survival Rate %		Median Survival Time (SE)	HR	95% CI for HR		*P*-value*
			2-year	4-year			Lower	Upper	
GCB	33	25	60.6	48.5	40 (16.7)	reference			
Non-GCB	72	61	23.0	10.5	14 (1.1)	2.29	1.400	3.750	*0.01*

[1] Subtypes by Hans algorithm; N, No. of patients; SE, standard error; HR, hazard ratio; CI, confidence level; *Cox regression, significant if P<0.05.

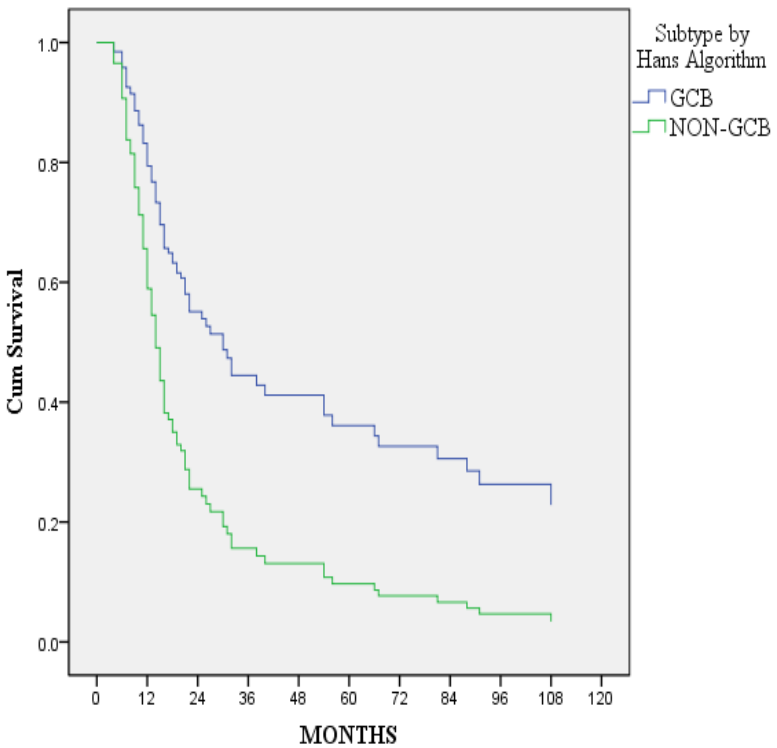

FIG. 3: Overall survival of patients with diffuse large B-cell lymphoma: Germinal centre B-cell (GCB) subtype versus non-germinal centre (non-GCB) subtype by Hans algorithm.

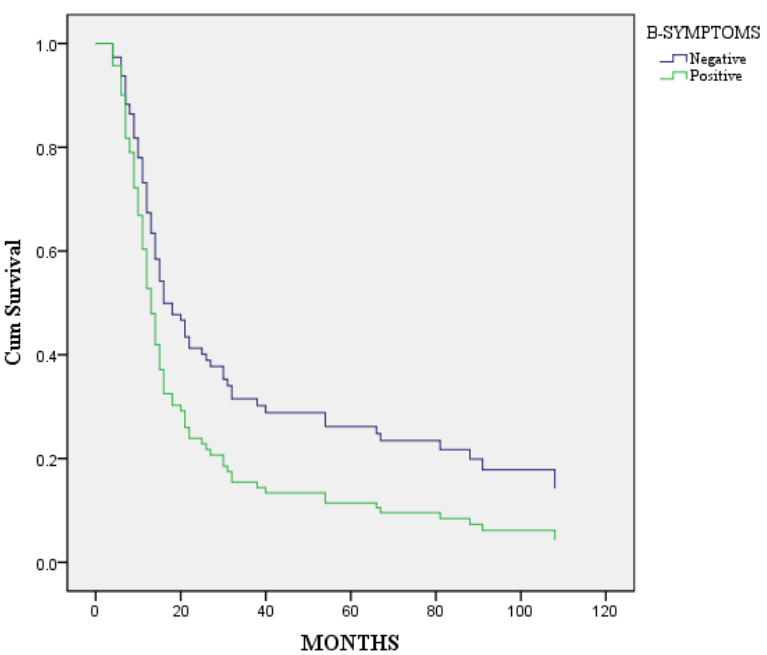

FIG. 4: Overall survival of diffuse large B-cell lymphoma patients with or without B-symptoms.

TABLE 9: Cox Regression Test (B symptoms)

B symptoms	N	No. of Death	Survival Rate %		Median Survival Time (SE)	HR	95% CI for HR		P-value*
			2-year	4-year			Lower	Upper	
Negative	75	56	35.6	31.2	29 (1.65)	Reference			
Positive	29	28	27.6	13.8	14 (1.05)	1.617	1.014	2.580	***0.037***

N, No. of patients; SE, standard error; HR, hazard ratio; CI, confidence level; * Cox regression, significant if P<0.05.

process to determine prognosis and to assist in making treatment decisions. These symptoms include fever, unexplained weight loss and drenching night sweats.

In our previous analysis by Chi Square Test, presence of B symptoms is one of the clinical parameters showed statistical difference in between GCB and non-GCB subtypes. The Kaplan-Meier curve indicates the association of B symptoms with the survival rate, P = 0.037. Out of 29 patients with the presence of B symptoms, 28 patients died of the disease. The curve revealed the overall 2-year and 4-year survival rates for B symptoms-positive patients are 27.6% and 13.8% respectively. The median survival time (the time at which 50% of patients have died) is 14 months (standard error= 1.05) for this group of patients. The Cox regression analysis showed that patients who have exhibited B symptoms are 62% more likely to die compared to those who did not have the symptoms (HR = 1.62), 95% CI, 1.014 – 2.580. Table 9 summarizes the Cox Regression Test for B symptoms and Figure 4 represents Kaplan-Meier curves for the analysis.

The Impact of Age on Survival of Diffuse Large B-Cell Lymphoma

We noticed that the survival experience is significantly different (P = 0.021) between two age groups (<60 and ≥60) by using log-rank test. Kaplan-Meier curve showed that patients with less than 60 years old have better survival outcome compared to the patients at 60 and older than 60 years old. The overall 2-year and 4-year survival rates for patients with <60 years old are 46.7% and 17.5% respectively whereas the survival rates for patients at ≥60 years old are 28.1% and 15.7% respectively. The median survival time (the time at which 50% patients have died) is 22 months (standard error= 8.1) for patients with <60 years old and 15 months (standard error= 1.1) for those with age at 60 and above. Cox regression analysis indicated that patients with age at 60 and above are 68% more likely to die compared to those who have with age below 60 (HR = 1.68, 95% CI, 1.07 – 2.64; P=0.021). Table 10 summarizes the Cox Regression Test for age and Figure 5 represents Kaplan-Meier curves for the analysis.

Survival Analysis of Serum Lactate Dehydrogenase

We explored the role of the serum lactate dehydrogenase (LDH) at initial diagnosis in the prognosis of diffuse large B-cell lymphoma. The cut-off values were determined by the recommendation from the manufacturer as well as verified by the local population study. We basically categorized our patients into three groups: ≤423 U/l, 424-1000 U/l and >1000 U/l. There were only 23.5% (28/119) patients showed a normal level of serum lactate dehydrogenase and 76.5% (91/119) patients with elevated serum lactate dehydrogenase above the upper limit of normal value. There was no association between the activity of this enzyme with the subtypes of

TABLE 10: Cox Regression Test (Age)

Age	N	No. of Death	Survival Rate %		Median Survival Time (SE)	HR	95% CI for HR		*P*-value*
			2-year	4-year			Lower	Upper	
<60	40	29	46.7	17.5	22 (8.1)	reference			
≥60	65	57	28.1	15.7	15 (1.1)	1.68	1.067	2.643	**0.021**

N, No. of patients; SE, standard error; HR, hazard ratio; CI, confidence level; * Cox regression, significant if P<0.05.

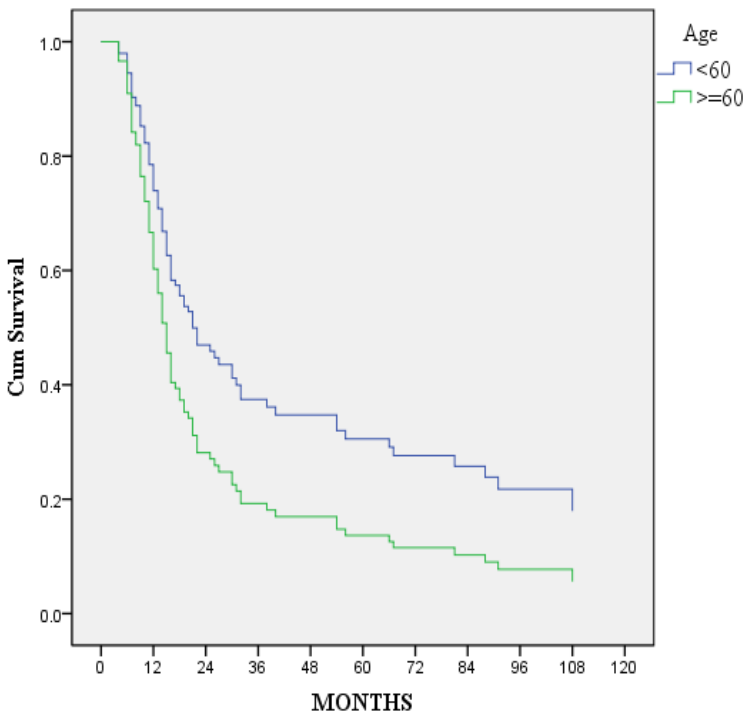

FIG. 5: Overall survival of patient group with age less than 60 and patient group at age of 60 and above.

the disease (P=0.192). In the survival analysis, log-rank (Mantel-Cox) indicated significant differences between the groups with P=0.012.

The overall 2-year and 5-year survival rates for patients with ≤423 u/l of serum LDH are 51.2% and 21.4% respectively whereas the survival rates for patients with LDH level at 424 – 1000 U/l are 38.5% and 19.9% respectively and lastly for the patients with LDH more than 1000 U/l are 23.1 and 0.0% respectively. The median survival time (the time at which 50% patients have died) is 25 months (standard error= 3.38) for patients with ≤423 u/l of serum LDH and 16 months (standard error= 2.07) for those with age at 424 – 1000 U/l and lastly 12 months for the patients with LDH level above 1000 U/l. Cox regression analysis indicated that patients with ≤423 U/l and above 1000 U/l of serum LDH have higher risk of dying due to the disease (424 – 1000 U/l, HR=1.1, 95% CI 0.6 – 2.06, P= 0.735; >1000 U/l, HR=2.2, 95% CI 1.15 – 4.14, P=0.016). Table 11 summarizes the Cox Regression Test for level of serum lactate dehydrogenase and Figure 6 represents Kaplan-Meier curves for the analysis.

CD5-positive DLBCL

CD5 expression was studied in 92 patients, 50 males and 42 females (ranged between 17-67 years). The median age of all patients in both CD5+ and CD5- groups was 62 and 61 years, respectively (Table 12). There were 11/92 (12%) CD5-positive cases and they were tested negative for cyclin D1, thus excluding the diagnosis of mantle cell lymphoma (Figure 7). This finding is in line with the previously published reports which indicated CD5 expression in 10 – 15% of DLBCL [17, 18]. Five of the cases were extranodal (colon, parotid gland, stomach and brain) (Table 13). It is noteworthy that 9 (81.8%) of CD5+ patients were categorized in the IPI high risk group and all died of the disease. There was no significant difference in patient basic characteristics between CD5-positive and CD5-negative groups. Of those 11 patients with CD5+, ten were categorized as non-GCB subtype (90.9% P=0.079).

Out of these 11 positive patients, 8 patients (72.7%) were treated with cyclophosphamide, doxorubicin (hydroxydaunomycin), vincristine and prednisolone plus rituximab (R-CHOP), 1 patient (9.1%) was treated with rituximab with Berlin-Frankfurt-Munster (BFM) therapy and 2 of them (18.2%) died before treatment was initiated (Table 13). CD5-positive patients showed significantly poorer outcome to treatment, regardless of rituximab, where 9 patients (81.8%) died of the disease. From our

TABLE 11: Cox Regression Test (LDH)

LDH*	N	No. of Death	Survival Rate %		Median Survival Time (SE)	HR	95% CI for HR		*P*-value*
			2-year	5-year			Lower	Upper	
<423	23	16	51.2	21.4	25 (3.38)	reference			
423-1000	40	29	38.5	19.9	16 (2.07)	1.1	0.6	2.061	0.735
>1000	26	26	23.1	0.0	12 (0.95)	2.2	1.15	4.14	**0.016**

*LDH, serum lactate dehydrogenase; N, No. of patients; SE, standard error; HR, hazard ratio; CI, confidence level; * Cox regression, significant if P<0.05.

data set, only ECOG performance status >1 is statistically significant where P = 0.011.

BCL2/MYC dual expression of DLBCL:

Double expressor lymphoma (DEL) refers to the DLBCL with both MYC and BCL2 protein overexpression detected by immunohistochemistry. Previous study indicated the importance of dual expression (DE) of MYC and BCL2 proteins as an independent indicator of poor prognosis in diffuse large B-cell lymphoma [19].

In our study, the cut-off points were 40% positive cells for MYC and 70% positive cells for BCL2, based on the previous studies [20, 21]. Tonsils with lymphoid hyperplasia were used as positive control tissues for both stains. The acceptable stain for BCL2 was characterized by the strong cytoplasmic staining in T-cells and none observed in germinal centre B-cells whereas MYC stain was characterized by a gradient of nuclear reactivity in lymphocytes (Figure 8).

A total of 29/70 (41.4%) cases were positive for MYC and 31/67 (46.3%) cases were positive for BCL2. There were merely 65 cases had results of both markers. Of these, 14/65 (21.5%) cases were positive for both MYC and BCL2 i.e. they were double expressors (DEs).

The Kaplan-Meir curves (Figure 9) were created to access both impact of MYC and BCL2 single expression respectively on the overall survival. Survival analysis by using log-rank test, showed no significance of single expression of MYC (P= 0.677) and BCL2 (P=

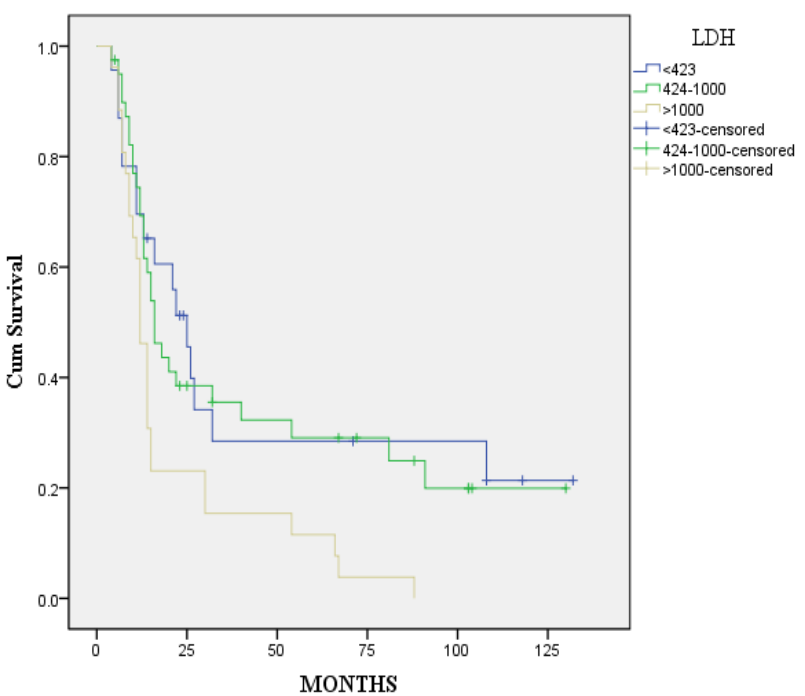

FIG. 6: Overall survival of patient group with different serum lactate dehydrogenase (LDH) levels.

TABLE 12: Demographic data of CD5+ DLBCL patients

	CD5 + DLBCL	CD5 - DLBCL	*P**
N	11 (12%)	81 (88%)	
Age			
Median	62	61.2	
Range	17-79	20-84	
<60	3	31	0.478
≥60	8	50	
Sex *(M:F)*	7:4	43:38	0.749
Stage *III/IV*	8/11 (72.7%)	50/81 (61.7%)	0.741
IPI[a]			
<3	2 (18.2 %)	24 (29.6 %)	0.311
≥3	9 (81.8 %)	40 (49.4 %)	
ECOG performance status			
>1	5 (45.5 %)	9 (11.1 %)	*0.011*
Tissue type			
Lymphatic	7	33	
Non-lymphatic	3	42	0.301
Brain	1	6	
Extranodal involvement more than 1 site	8 (72.7 %)	39 (48.2 %)	0.199
Subtypes			
GCB	1 (9.1 %)	26 (31.0 %)	
Non-GCB	10 (90.9 %)	47 (69.0 %)	0.079
Status			
Alive	2 (18.2 %)	24 (29.6 %)	
Died	9 (81.8 %)	57 (70.4 %)	0.722

IPI *international prognostic index*, GCB *germinal centre B-cell like;* * P<0.05; [a] Cases excluded due to incomplete clinical data; *italics* represent statistically significant.

TABLE 13: Sites of specimens, treatment and survival details in CD5-positive DLBCL

No.	Extranodal/ Nodal	Site of specimens	Age/Sex	Treatment	Survival Status
1	Extranodal	Brain	67/M	Rituximab + BFM	Alive
2	Nodal	Supraclavicular lymph node	79/F	RCHOP	Alive
3	Nodal	Supraclavicular lymph node	60/M	RCHOP	Died
4	Nodal	Lymph node	58/M	RCHOP	Died
5	Nodal	Tonsil	68/M	RCHOP	Died
6	Extranodal	Parotid gland	17/F	Died before initiate chemo	Died
7	Nodal	Cervical lymph node	52/M	RCHOP	Died
8	Extranodal	Gastro biopsy	68/F	RCHOP	Died
9	Nodal	Mesentric lymph node	70/F	Died before initiate chemo	Died
10	Extranodal	Colon	69/M	RCHOP	Died
11	Extranodal	Tonsil	74/M	RCHOP	Died

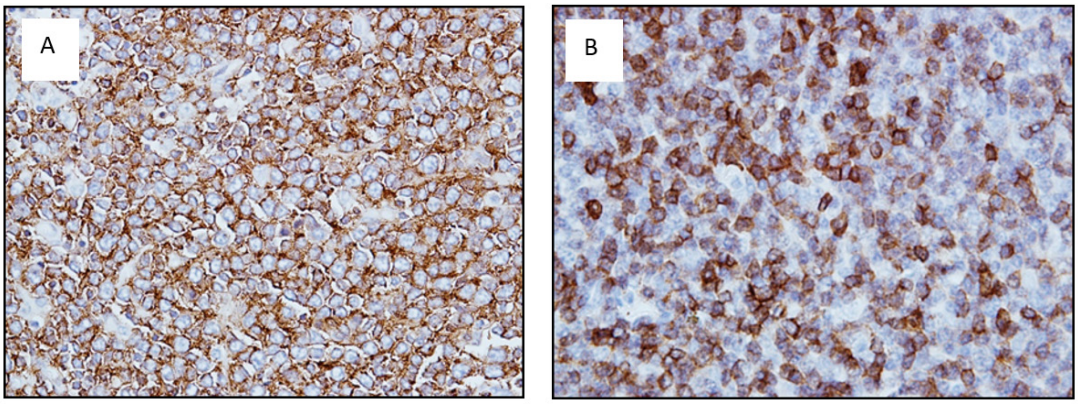

FIG. 7: The expression of CD5 by immunohistochemistry on the membrane surface of tumour cells (X400); A: Diffuse large B-cell lymphoma, B: Mantle cell lymphoma.

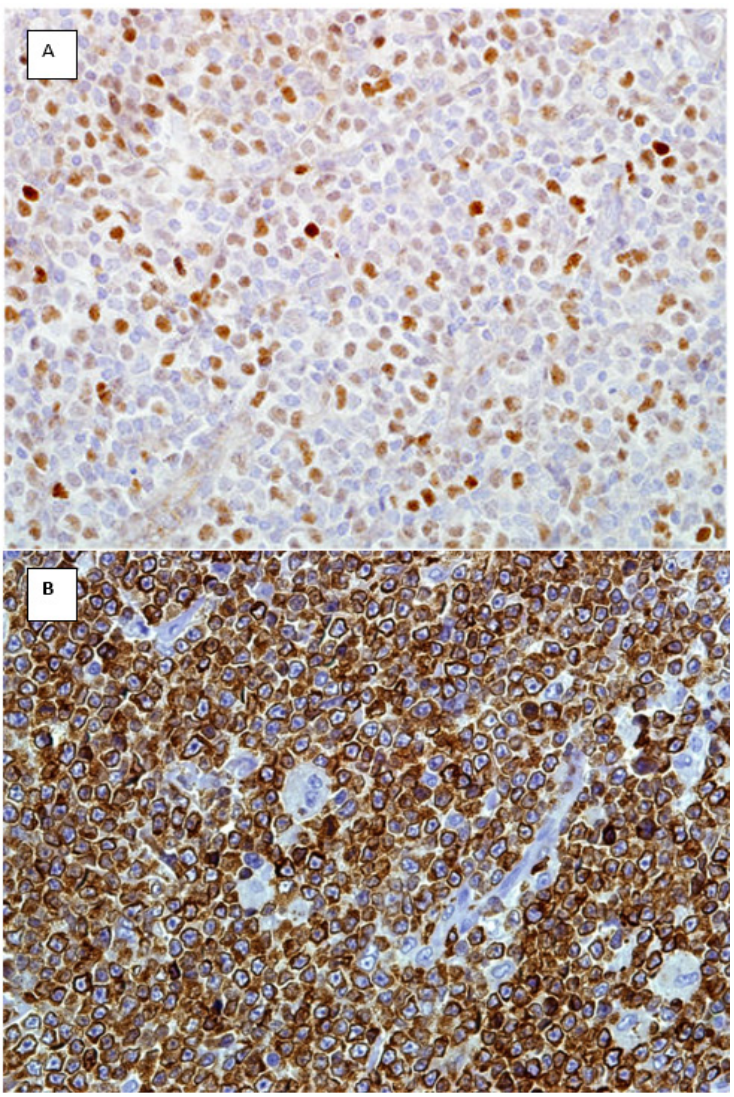

FIG. 8: Immunohistochemical staining of A: MYC and B: BCL2 (X400). Note that the variation in staining intensity of MYC is still considered positive.

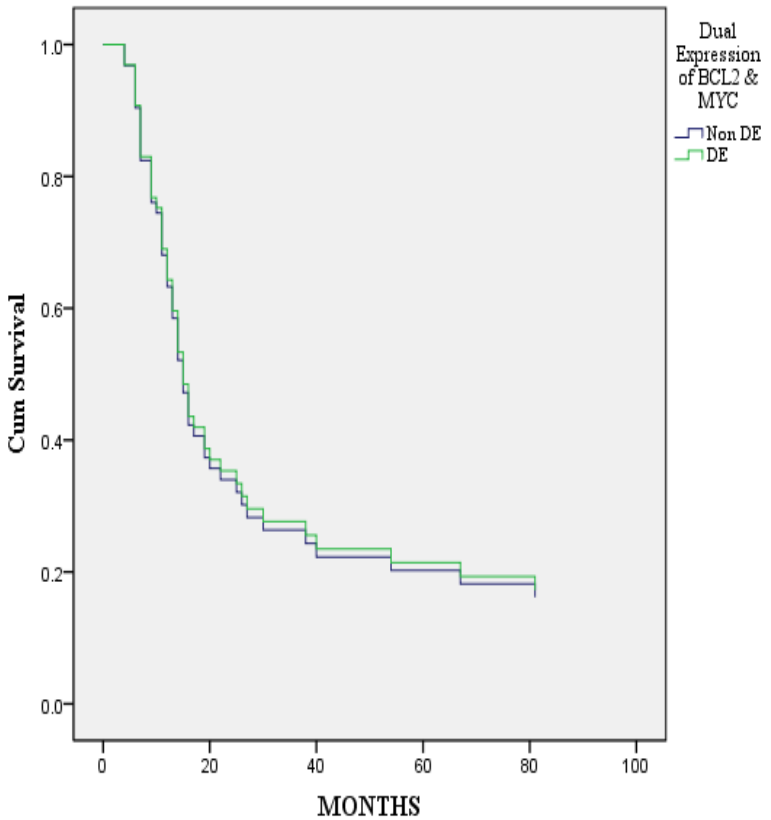

FIG. 9: Overall survival of diffuse large B-cell lymphoma patients with or without dual expression of MYC and BCL2 proteins.

0.681) in our population. Double expression of MYC and BCL2 proteins yielded no significant difference in overall survival compared to non-double expression cases (P = 0.916) with overall 2-year and 4-year survivals of 46.2% and 23.1% respectively (HR = 0.965; CI 95% 0.493, 1.888) (Table 14).

Findings of fluorescent *in situ* hybridization (FISH):

Detection of Gene Rearrangement Using Tissue Microarray

FISH analysis using split-apart probes for *BCL-2*, *BCL-6*, *BCL-10*, *MALT-1* and *MYC* genes (Dako, Denmark) was performed to determine their status in diffuse large B-cell lymphoma. In sum, 13 cases (9.22 %) out of 141 showed the presence of gene rearrangements. Figure 10 and Figure 11 show the examples of FISH analyses performed on formalin fixed paraffin embedded (FFPE) section. The overall gene rearrangements are summarized in Table 15.

BCL-6 exhibited the highest frequency of rearrangements (76.9%), followed by two cases for *MYC* (15.4%) and one case for *BCL-2* (7.7%) genes.

No *BCL-10* and *MALT-1* gene rearrangements were found in this study. Multiple rearrangements were not detected in any case.

TABLE 14: Cox Regression Test (Dual expression of MYC and BCL2)

DE	N	No. of Death	Survival Rate %		Median Survival Time (SE)	HR	95% CI for HR		*P*-value*
			2-year	4-year			Lower	Upper	
Positive	14	12	46.2	23.1	16 (7.79)	0.965	0.493	1.888	0.916

DE, dual expression of MYC and BCL2; N, No. of patients; SE, standard error; HR, hazard ratio; CI, confidence level; * Cox regression, significant if P<0.05.

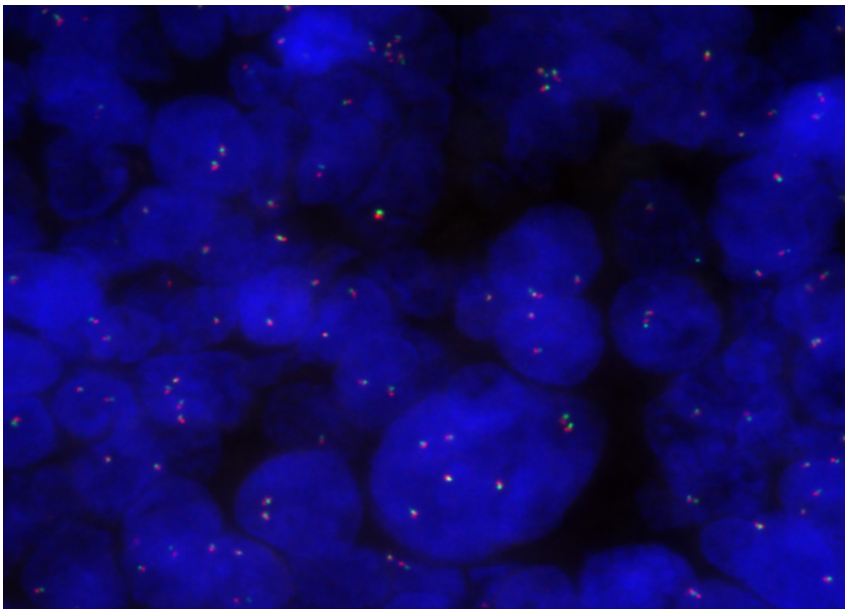

FIG. 10: Normal signals (Split signal BCL2 probe)

Beside the rearrangements observed, 3 cases were identified with the presence of trisomy pattern (2.1%), whereas 6 cases showed polysomy pattern (4.3%) and 2 cases with amplification pattern (1.4%).

Detection of Gene Rearrangement Using Whole Formalin Fixed Paraffin Embedded (FFPE) Tissue Sections

Interestingly, the number of cases with gene rearrangements were lower than the published reports. Hence, we undertook to validate the results using whole FFPE tissue sections with the same methodology and materials. However, there were only three additional *BCL6* gene rearranged cases found using this method, increasing the total number of gene rearranged cases to 16 (11.35%) out of 141 cases. By using Cohen's kappa statistical analysis, we obtained perfect agreement for both *BCL2* and *MYC* (κ=1)

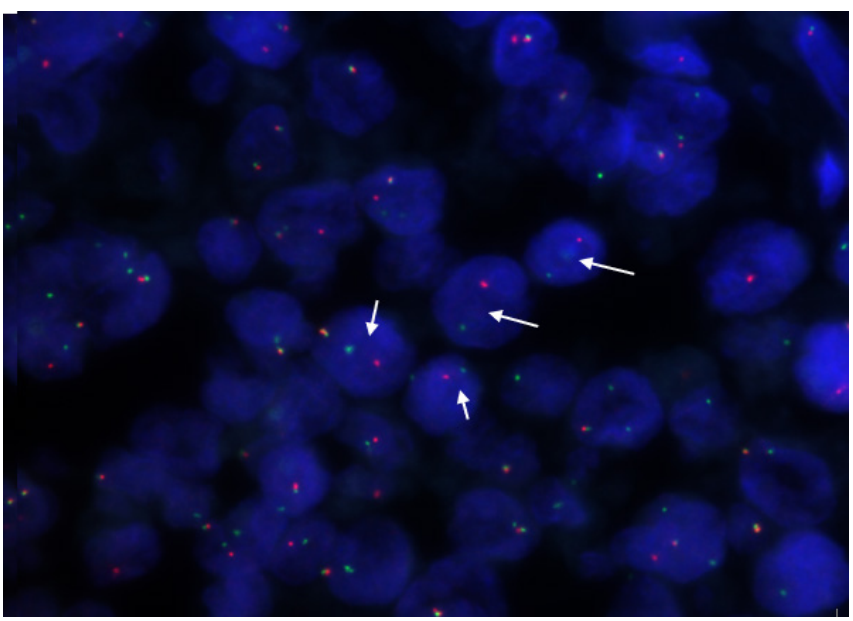

FIG. 11: Split signals demonstrate the presence of rearrangements of BCL6

TABLE 15: Gene rearrangements detected in 141 cases of DLBCL using a panel of split-signal FISH probes

Probe	Total	Percentage (%)
BCL6	10+3 (additional from whole section)	81.25
BCL 2	1	6.25
BCL10	0	0
MALT-1	0	0
MYC	2	12.50

as there was no difference in outcome using either TMA or whole sections. As for *BCL6*, the kappa value obtained was 0.853 and this fulfilled substantial agreement according to the commonly cited scale described[16]. We did not find case with double gene rearrangements that is double-hit lymphoma.

DISCUSSION

Diffuse large B-cell lymphoma (DLBCL) is an aggressive type of non-Hodgkin's lymphoma which is the most common lymphoid malignancy accounting for thirty to forty percent of all lymphoma cases across different geographic regions. The incident rate of DLBCL is approximately 7 per 100,000 as found in the United States National Cancer Institute's SEER Database and age-standardized incident rate is estimated at 5.0 per 100,000 people as per reported by the International Agency for Research on Cancer (IARC).[22] This disease exhibits heterogeneous clinical behaviour with unpredictable and highly variable outcome and it can grow rapidly and become fatal if the patients are left untreated. In view of the pattern and frequency of DLBCL that vary in different geographical regions as well as populations, the aims of this Malaysian study were to explore the demographic characteristics, immunophenotypic profiling of diffuse large B-cell lymphoma in depth and its association with survival, the status of gene rearrangement in terms of translocation, as well as to validate the current practice of immunohistochemistry in the routine setting. The findings presented herein can be used as an improved guideline in laboratory investigation for this high-grade non-Hodgkin's lymphoma.

A total of 141 cases of diffuse large B-cell lymphoma which were collected over a period of seven years (2004 – 2010) was recruited. In these cases, there were 81 (57.4%) cases of lymphoid tissues, 52 (36.9%) non-lymphoid tissues, and eight (5.7%) brain tissues. All cases were reviewed by experienced haematopathologists and they had no history of other lymphoproliferative disorders. The ratio of female to male occurrence was 1:1.27 which showed the male predominance for the disease and this finding is in line with previous studies.[23,24]

Being more than 60 years old was identified as one of the adverse risk factors for DLBCL patients, at which one of the largest population-based studies by Gustaf *et al*. indicated prominent significance of older age on the survival rate[25,26]. Our study on age showed a statistically significant difference in age groups of <60 and ≥ 60, P = 0.021, in which patients aged 60 and above have 1.68 (95% CI: 1.01, 2.64) higher odds of contracting the disease compared to those aged below 60. Between the subtypes, it was found that patients below 60 years old are more likely to be categorised as GCB compared to those 60 years and above (OR = 2.40; 95% CI: 1.07, 5.37; P = 0.03) at which patients with non-GCB have an inferior outcome of the treatment. The cause of shorter survival with increasing age remains unclear though there were several studies that found the biological factors contribute to the inferior outcome with increased age.[27-29] Another reason is the co-morbidities associated with elderly patients which in turn making the treatment even more difficult to take place. Also, toxicity that built up in elderly patients often causes the premature cease of treatment.[30]

Malaysia is a multiracial country consisting of Malays, Chinese, Indians, and other ethnics; therefore, it would be interesting to explore the relationship of races with the heterogeneity of the disease. There were a few well-established studies which previously reported significant racial differences in NHL and DLBCL.[31-33] However, our result found no significant racial differences in survival, P = 0.109. From the logistic regression analysis comparing GCB and non-GCB subtypes, it was postulated that the Chinese had 1.51 (95% CI, 0.635, 3.58) higher odd compared to the Malays though the result

was not statistically significant (P = 0.352). It is notable that other ethnic groups showed a significant outcome with P = 0.04, OR = 0.098 (95% CI 0.011, 0.892) which meant that they were less likely to be categorised as non-GCB subtypes in comparison to the Malays. A better picture for this analysis can be acquired by increasing the sample size as in this study, only one Indian patient and five patients from other ethnic groups were recruited.

B symptoms (also known as disease-associated systemic symptoms) refer to constitutional symptoms that are associated with both Hodgkin's lymphoma and non-Hodgkin's lymphoma, which include high fever without infection, night sweats or sometimes drenching sweats, unexplained weight loss of at least 10% of the normal body weight, in some cases with the presentation of pain in bones, chest, and abdomen as well as experiencing chronic fatigue. DLBCL patients with the presence of B symptoms were labelled with the letter 'B' behind the stage level as well, which reflects the prognostic significance of these symptoms. Our results on the presence of B symptoms showed significant differences between GCB and non-GCB subtypes (P = 0.034) as well as the survival rates (P = 0.037) with a higher risk of dying for those that presented B symptoms (HR = 1.617, 95% CI 1.014, 2.580). However, the limitation for this part of the study was that the patients without B symptoms were twice more than those with the symptoms. It is therefore suggested that having relatively similar sample sizes for both groups is better to acquire highly representative results of the analysis. From the present study, the inferior outcome of the patients that present B symptoms could be predicted.

The immunohistochemical study on diffuse large B-cell lymphoma

Immunohistochemistry (IHC) is a valuable tool that, in the last four decades, has revolutionised the practice of diagnostic histopathology and haematopathology, as well as in research purpose to enhance the current characterisation of tumours and risk stratification. There were in total six tissue microarray (TMA) blocks constructed using 141 formalin-fixed paraffin embedded tissue samples from the Department of Pathology, UKM Medical Centre, with two replicates for each block. All cases were reviewed, and specific tumour areas were selected by experienced histopathologists prior to the TMA construction. By using standard staining protocols which included the control tissues, the immunohistochemical staining of 10 markers were evaluated; namely, CD10, BCL6, MUM1, GCET1, FOXP1, CD5, Ki67, CD20, MYC and BCL2 and between GCB and non-GCB. The results were significantly different for CD10 (P < 0.01), BCL6 (P < 0.01), and MUM1 (P = 0.02), in which, they are the essential stains for Hans algorithm.

CD20 is a non-glycosylated phosphoprotein expressed on the surface of B-cell at all stages of development except for the first and last stages. It is widely used for differential diagnosis of B-cell and T-cell lymphomas such as diffuse large B-cell lymphoma (DLBCL) and anaplastic large cell lymphoma (ALCL) which are morphologically identical under the microscope. Therefore, monoclonal antibodies for instance, rituximab and ibritumomab tiuxetan were utilised for efficient target therapy in the treatment of B-cell lymphoma as they recognise those CD20 antigens on the cellular surface. This study indicated high incidence of positive CD20 DLBCL, with 91.1% (102/122) were positive in CD20 expression. There was no statistical significance between the subtypes of the disease (P = 0.950). However, it is notable that all CD20-negative DLBCL patients died during the follow up period with 2-year overall survival at 22.2% though the results were not statistically significant (P = 0.544) by the mean of the log-rank test. The finding was similar to other published studies,[34,35] showing that inferior outcome associated with the reduced expression of CD20 and the finding of IHC results should be correlated with the results by flow cytometry.

Determination of Molecular Subtypes of Diffuse Large B-cell Lymphoma (GCB and non-GCB)

In the next part of the study, a comparison study for both subtyping results was performed using the well-established Hans and Choi immunohistochemical algorithms. As for Hans algorithm, the samples were stained with CD10, BCL6, and MUM1 antibodies and additional GCET1 and FOXPI antibodies for Choi algorithm. Out of 141 cases, the results of 80.1% (113/141) for Hans algorithm and 78% (110/141) for Choi algorithm were acquired. Cohen's kappa analysis indicated the strong agreement of the two algorithms, $\kappa = 0.828$ (95% CI), P < 0.001. Therefore, we can conclude that both algorithms produced highly concordant results in our routine laboratory.

There were many studies which reported respective findings on the prognostic implications of cell-of-origin in patients with diffuse large B-cell lymphoma (DLBCL). Table 16 shows various studies on subtypes of DLBCL by Hans and Choi algorithms reported from distinguished geographic regions, including International, Western, China, Korea, Taiwan, Japan, Spain, as well as Malaysia. Interestingly, except for Japan, the current finding on the proportion of non-GCB and GCB subtypes was relatively similar to other Asian countries: China, Korea, and Taiwan, indicating that geographical variations and genetic differences between Western and Asian populations may contribute to different patterns of the disease manifestation. Malaysia has a relatively larger proportion of patients (67.3%) with non-GCB subtype of diffuse large B-cell lymphoma compared to the Western countries and therefore, explains the differences of our research outcomes compared to those reported by Western studies.

By using the Kaplan Meir method and the log-rank test as well as proportional hazards regression, it is noticed that patients with non-GCB subtype had relatively unfavourable prognosis than those with GCB subtype (HR = 2.29, 95% CI, 1.4 – 3.75; 4-year OS, 10.5%; $P < 0.001$) and age > 60-years-old, races, poor R-IPI, CD5-positive DLBCL and the presence of B symptoms were more distributed in non-GCB DLBCL as well. The observation was similar to many published reports, stating that non-GCB subtype is an adverse prognostic factor [36-38]. Statistically significant results were acquired in the scenario of either using Hans ($P < 0.001$) or Choi algorithm ($P = 0.021$), but Hans algorithm had shown a stronger prognostic predictive value. Thus, similar to previous research, the finding proves unequivocally the usefulness of Hans algorithm as an independent prognostic tool for diffuse large B-cell lymphoma and it helps to identify the patients who need an aggressive therapy.[39]

Expression of CD5 in Diffuse Large B-cell Lymphoma

CD5 is a pan-T-cell marker typically expressed on T cells and a subset of normal naïve B-cells, as well as lymphoma such as chronic lymphocytic leukaemia/small lymphocytic lymphoma (CLL/SLL) and mantle cell lymphoma but rarely expressed in diffuse large B-cell lymphoma. To date, many CD5 DLBCL studies were conducted in large-scale series in Japan [40-47] but not in Malaysia. Therefore, it would be interesting to know the incidence of CD5-positive DLBCL in Malaysia along with its clinical significance.

In the current study of CD5-positive diffuse large B-cell lymphoma, a total of 92 evaluable formalin-fixed paraffin embedded (FFPE) archival samples of diffuse large B-cell lymphoma (DLBCL) from the Department of Pathology, Universiti Kebangsaan Malaysia Medical Centre were tested. A panel of antibodies namely CD5, CD3, cyclin D1, CD10, BCL6, and MUM1 (Dako, Denmark) were used for immunohistochemical staining. CD3 and cyclin D1 stainings were performed to exclude the possibility of Richter's transformation (RT) of SLL/CLL as well as mantle cell lymphoma as both are also CD5 expressing B-cell lymphomas. CD10, BCL6, and MUM1 stainings were utilised to divide the samples into GCB and non-GCB subtypes in accordance to Hans algorithm.

Consequently, CD5+ DLBCL comprised 12% of all 92 DLBCLs in the current study of the Malaysian population and this finding appeared to be in keeping with those of the previous reports [47-50] which stated the incidence

TABLE 16: Frequency of DLBCL with non-GCB subtype in various geographical regions by Hans algorithm.

Country/region (n)	Non-GCB, %	Reference
International (431)	43.9	(56)
West (169)	53.3	(57)
China (63)	67	(58)
Taiwan (153)	72.5	(59)
Korea (170)	69.4	(60)
Japan (730)	51.8	(61)
Spain (149)	59.1	(62)
Malaysia (141)	67.3	This study

of 10 – 15% CD5+ DLBCL. Interestingly, this study showed that CD5+ DLBCL predominantly affected elderly people; many patients tended to be associated with poor prognostic components of IPI, extranodal involvement and advanced clinical stage (III/IV) and 90.9% (10/11) CD5-positive DLBCL cases were distributed in non-GCB subtype which is associated with inferior outcome of treatment. The prognosis of CD5+ DLBCL was significantly poorer than CD5- DLBCL as 81.8% of patients were categorised in the high risk IPI group. This remarkable finding suggested that CD5 expression is a potential prognostic biomarker in a clinical diagnostic setting.

In accordance to the Nordic Lymphoma Study Group, CD5-positive DLBCLs were associated with inferior OS and failure-free survival.[51] A previous study also showed 34% of 5-year survival rate for CD5-positive cases treated with anthracycline-based regimen.[40] In the present study, it was found that CD5-positive cases showed significantly poorer survival rate in which 9 patients (81.8%) with CD5 expression died of the disease and the 2-year survival rate was 42.9%. As for between the subtypes as well as the survival rate, the analyses indicated borderline significance with P = 0.079 (subtypes) and P = 0.076 (survival). Therefore, this study showed the importance of CD5 expression as one of the potential predictors of poor outcome (P = 0.076). A multicentred validation study with a larger scale of samples especially more CD5-positive cases should be carried out in order to obtain a more representative result for future studies in Malaysia. The assessment of this biomarker should be included as per recommended in the 2016 update of the World Health Organization (WHO) classification of DLBCL.[52]

Expression of MYC and BCL2 in Diffuse Large B-cell Lymphoma

Studies have shown that in lymphoma cases with overexpression of both BCL2 and MYC proteins which are normally recognised as double-expressor lymphoma (DEL) have long been known to confer inferior prognosis compared to those with one or neither of these overexpressions. However, the optimal cutoffs to define overexpression of MYC and BCL2 are not well identified for the Malaysian population. Past studies had used different BCL2 and MYC cut-offs of ≥70% and ≥40%; ≥50% and ≥40%, and ≥30% and ≥50% respectively [53-55]. We used cutoffs of ≥70% for BCL2 and ≥40% for MYC in this study and the results found were not statistically significant for survival analysis with P = 0.916. Although there was no significant impact on the subtypes, bivariate regression logistic analysis indicated that DELs were predominantly non-GCB subtype (OR = 1.72; 95% CI 0.478, 6.184; P = 0.407). The results suggested that the double expression of BCL2 and MYC did not affect the survival outcome of the disease. However, further studies are required to confirm these results with a few important aspects to be emphasised. Firstly, the study sample size should be increased as in this present study, due to lack of results from tissue microarray on the stainings of MYC and BCL2, merely 48.2% (68/141) cases were identified for double-expressor (DE) and non-double-expressor (non-DE). The other limitation was the uncertainty regarding IHC scoring thresholds for these two markers. More recently, papers showed that Johnson *et al*. successfully identified DEL patients with poor survival by using a cutoff of ≥50%/40%.[54] It is then suggested that a larger series of uniformly treated cases be tested with different cut-offs and the validity as well as the reliability of the results be further investigated.

Gene rearrangements in diffuse large B-cell lymphoma in Malaysians using tissue microarray and whole formalin-fixed paraffin embedded sections

In the FISH study, 141 formalin-fixed paraffin embedded (FFPE) samples of diffuse large B-cell lymphomas were tested using a panel of split signals FISH probes (Dako, Denmark); namely, *BCL2, BCL6, BCL10, MYC,* and *MALT-1*. All duplicated tissue microarray (TMA) samples were run together with control tissues such as placentas and normal lymph nodes to ensure the reliability of the experiments.

In the present series of specimens, a mere 9.22% of cases, (13/141 cases) presented gene-specific abnormality. The most involved gene was *BCL6* which consisted of 76.9% of the total rearrangement cases. Interestingly, this finding corresponds with the results reported in a previous testing done in Europe.[56] However, there was a relatively lower incidence of rearrangements for both *BCL2* and *MYC* genes, while none was found in *BCL10* and *MALT-1*. Furthermore, none of the cases showed more than one gene rearrangement. Hence, the results were further validated using the whole section of FFPE samples with the same optimised method in which specific tumour

areas were studied thoroughly. However, the outcome was almost similar to the one done on TMAs which were much smaller than the ones in the whole histologic slides. Since there was a very low number of cases that exhibited gene rearrangements, a conclusive finding could not be drawn about the correlation of this genetic abnormality with the subtypes (germinal centre type and non-germinal centre type), clinical outcome, as well as the revised International Prognostic Index (R-IPI) data.

It is important to note that all MYC gene rearrangement positive cases have increased MYC protein. In comparison with the results of immunohistochemical staining, there were merely two cases of IHC positive cases which showed 8q24 translocation involving *MYC* gene, suggesting that there were other mechanisms contributing to the overexpression of MYC protein. Cases with the presence of gene rearrangement and protein overexpression were associated with aggressive clinical behaviour in which all patients died of the disease.

It is recommended that in order to make this study more representative, there is a need to continuously collect more cases for these testings. Besides, FISH analysis may be performed on separate nuclei extracted from the sample cores since this method is likely to secure more conclusive findings in the detection of gene rearrangements.

CONCLUSION

In the present study, we have found that large proportion of our DLBCL patients presented non-GCB subtypes in comparison to Western countries. We presented a few useful prognostic factors throughout the study, including age, B symptoms, serum lactate dehydrogenase and subtypes that bring significant impacts to the prognosis of the disease. We provided additional supportive evidence that Hans algorithm is an essential prognostic tool for diffuse large B-cell lymphoma. Besides, we discovered 12% of the DLBCL cases expressed CD5 protein which had shown adverse outcome of treatment. However, in our FISH analysis, we acquired relatively low incidence of gene arrangements. It is therefore mandatory to incorporate the study into a bigger context and investigate all aspects of the disease in order to understand comprehensively the biology of diffuse large B-cell lymphoma in the Malaysian population, from epidemiological study to molecular characteristics.

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REFERENCES

1. Azizah Ab M NSIT, Noor Hashimah A, Asmah Z.A, Mastulu W. Malaysian National Cancer Registry Report 2007 - 2011. 2016.
2. Blom B, Human B, Anagnostou D, Matutes E. Bone Marrow Lymphoid Infiltrates: Springer Science & Business Media; 2012. 285 p.
3. Choi WWL, Weisenburger DD, Greiner TC, *et al*. A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009; 15(17): 5494-502.
4. de Leval L, Harris NL. Variability in immunophenotype in diffuse large B-cell lymphoma and its clinical relevance. Histopathology. 2003; 43(6): 509-28.
5. Kluk MJ, Chapuy B, Sinha P, *et al*. Immunohistochemical detection of MYC-driven diffuse large B-cell lymphomas. PloS one. 2012; 7(4): e33813.
6. Said J. Diffuse aggressive B-cell lymphomas. Advances in anatomic pathology. 2009; 16(4): 216-35.
7. Hans CP, Weisenburger DD, Greiner TC, *et al*. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004; 103(1): 275-82.
8. Chaganti SR, Chen W, Parsa N, *et al*. Involvement of BCL6 in chromosomal aberrations affecting band 3q27 in B-cell non-Hodgkin lymphoma. Genes Chromosomes Cancer. 1998; 23(4): 323-7.
9. Kramer MH, Hermans J, Wijburg E, *et al*. Clinical relevance of BCL2, BCL6, and MYC rearrangements in diffuse large B-cell lymphoma. Blood. 1998; 92(9): 3152-62.
10. Muramatsu M, Akasaka T, Kadowaki N, Ohno H, Fukuhara S, Okuma M. Rearrangement of the BCL6 gene in B-cell lymphoid neoplasms. Leukemia. 1997;11 Suppl 3: 318-20.
11. Offit K, Louie DC, Parsa NZ, *et al*. BCL6 gene rearrangement and other cytogenetic abnormalities in diffuse large cell lymphoma. Leuk Lymphoma. 1995; 20(1-2): 85-9.
12. Sanchez-Izquierdo D, Buchonnet G, Siebert R, *et al*. MALT1 is deregulated by both chromosomal translocation and amplification in B-cell non-

Hodgkin lymphoma. Blood. 2003; 101(11): 4539-46.
13. Pileri SA, Roncador G, Ceccarelli C, *et al*. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. J Pathol. 1997; 183(1): 116-23.
14. Sehn LH, Berry B, Chhanabhai M, et al. The revised International Prognostic Index (R-IPI) is a better predictor of outcome than the standard IPI for patients with diffuse large B-cell lymphoma treated with R-CHOP. Blood. 2007; 109(5): 1857-61.
15. Ashby D. Practical statistics for medical research. Douglas G. Altman, Chapman and Hall, London, 1991. No. of pages: 611. Price: £32.00. Statistics in Medicine. 1991; 10(10): 1635-36.
16. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977;33(1): 159-74.
17. Xu-Monette ZY, Tu M, Jabbar KJ, *et al*. Clinical and biological significance of de novo CD5+ diffuse large B-cell lymphoma in Western countries. Oncotarget. 2015; 6(8): 5615-33.
18. Yamaguchi M, Seto M, Okamoto M, *et al*. De novo CD5+ diffuse large B-cell lymphoma: a clinicopathologic study of 109 patients. Blood. 2002; 99(3): 815-21.
19. Scott DW, Mottok A, Ennishi D, *et al*. Prognostic Significance of Diffuse Large B-Cell Lymphoma Cell of Origin Determined by Digital Gene Expression in Formalin-Fixed Paraffin-Embedded Tissue Biopsies. J Clin Oncol. 2015; 33(26): 2848-56.
20. Green TM, Young KH, Visco C, *et al*. Immunohistochemical double-hit score is a strong predictor of outcome in patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol. 2012; 30(28): 3460-7.
21. Horn H, Ziepert M, Becher C, *et al*. MYC status in concert with BCL2 and BCL6 expression predicts outcome in diffuse large B-cell lymphoma. Blood. 2013; 121(12): 2253-63.
22. Teras LR, DeSantis CE, Cerhan JR, Morton LM, Jemal A, Flowers CR. 2016 US lymphoid malignancy statistics by World Health Organization subtypes. CA Cancer J Clin. 2016.
23. Fadilah SA. Fundamentals of the management of non-Hodgkin lymphoma. Med J Malaysia. 2009; 64(4): 333-9; quiz 40.
24. Yildirim M, Kaya V, Demirpence O, Paydas S. The role of gender in patients with diffuse large B cell lymphoma treated with rituximab-containing regimens: a meta-analysis. Arch Med Sci. 2015; 11(4): 708-14.
25. Brenner H, Gondos A, Pulte D. Ongoing improvement in long-term survival of patients with Hodgkin disease at all ages and recent catch-up of older patients. Blood. 2008; 111(6): 2977-83.
26. Hedström G, Hagberg O, Jerkeman M, Enblad G. The impact of age on survival of diffuse large B-cell lymphoma – a population-based study. Acta Oncologica. 2015; 54(6): 916-23.
27. Klapper W, Kreuz M, Kohler CW, *et al*. Patient age at diagnosis is associated with the molecular characteristics of diffuse large B-cell lymphoma. Blood. 2012; 119(8): 1882-7.
28. Mareschal S, Lanic H, Ruminy P, Bastard C, Tilly H, Jardin F. The proportion of activated B-cell like subtype among de novo diffuse large B-cell lymphoma increases with age. Haematologica. 2011; 96(12): 1888-90.
29. Thunberg U, Amini RM, Linderoth J, Roos G, Enblad G, Berglund M. BCL2 expression in de novo diffuse large B-cell lymphoma partly reflects normal differences in age distribution. Br J Haematol. 2009; 146(6): 683-4.
30. Boslooper K, Kibbelaar R, Storm H, *et al*. Treatment with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone is beneficial but toxic in very elderly patients with diffuse large B-cell lymphoma: a population-based cohort study on treatment, toxicity and outcome. Leukemia & Lymphoma. 2014; 55(3): 526-32.
31. Flowers CR, Shenoy PJ, Borate U, *et al*. Examining racial differences in diffuse large B-cell lymphoma presentation and survival. Leuk Lymphoma. 2013;54(2): 268-76.
32. Li Y, Wang Y, Wang Z, Yi D, Ma S. Racial differences in three major NHL subtypes: descriptive epidemiology. Cancer Epidemiol. 2015; 39(1): 8-13.
33. Shenoy PJ, Malik N, Nooka A, *et al*. Racial differences in the presentation and outcomes of diffuse large B-cell lymphoma in the United States. Cancer. 2011; 117(11): 2530-40.
34. Johnson NA, Boyle M, Bashashati A, *et al*. Diffuse large B-cell lymphoma: reduced CD20 expression is associated with an inferior survival. Blood. 2009;113(16): 3773-80.
35. Jo J, Yoo C, Ahn Y, *et al*. Comparison Between CD20-Negative and CD20-Positive Diffuse Large B Cell Lymphoma; Characteristics and Clinical Outcome. Blood. 2010; 116(21): 4891-91.
36. Wilson WH. Treatment strategies for aggressive lymphomas: what works? Hematology Am Soc Hematol Educ Program. 2013;2013: 584-90.
37. Lenz G, Staudt LM. Aggressive lymphomas. N Engl J Med. 2010; 362(15): 1417-29.
38. Habara T, Sato Y, Takata K, *et al*. Germinal center B-cell-like versus non-germinal center B-cell-like as important prognostic factor for localized nodal DLBCL. J Clin Exp Hematop. 2012; 52(2): 91-9.
39. Jablonska J, Jesionek-Kupnicka D, Potemski P, Kowalik A, Sygut J, Kordek R. Comparison of two different immunohistochemical algorithms identifying prognostic subgroups of DLBCL. Pol J Pathol. 2010; 61(3): 124-32.
40. Yamaguchi M, Seto M, Okamoto M, *et al*. De novo CD5+ diffuse large B-cell lymphoma: a clinicopathologic study of 109 patients. Blood. 2002; 99(3): 815-21.
41. Yamaguchi M, Ohno T, Oka K, *et al*. De novo CD5-positive diffuse large B-cell lymphoma: clinical characteristics and therapeutic outcome. Br J Haematol. 1999; 105(4): 1133-9.
42. Yamaguchi M, Nakamura N, Suzuki R, *et al*. De novo CD5+ diffuse large B-cell lymphoma: results of a detailed clinicopathological review in 120

patients. Haematologica. 2008; 93(8): 1195-202.
43. Niitsu N, Okamoto M, Tamaru JI, *et al*. Clinicopathologic characteristics and treatment outcome of the addition of rituximab to chemotherapy for CD5-positive in comparison with CD5-negative diffuse large B-cell lymphoma. Ann Oncol. 2010; 21(10): 2069-74.
44. Miyazaki K, Yamaguchi M, Suzuki R, *et al*. CD5-positive diffuse large B-cell lymphoma: a retrospective study in 337 patients treated by chemotherapy with or without rituximab. Ann Oncol. 2011; 22(7): 1601-7.
45. Hyo R, Tomita N, Takeuchi K, *et al*. The therapeutic effect of rituximab on CD5-positive and CD5-negative diffuse large B-cell lymphoma. Hematol Oncol. 2010;28(1): 27-32.
46. Harada S, Suzuki R, Uehira K, *et al*. Molecular and immunological dissection of diffuse large B cell lymphoma: CD5+, and CD5- with CD10+ groups may constitute clinically relevant subtypes. Leukemia. 1999; 13(9): 1441-7.
47. Ennishi D, Takeuchi K, Yokoyama M, *et al*. CD5 expression is potentially predictive of poor outcome among biomarkers in patients with diffuse large B-cell lymphoma receiving rituximab plus CHOP therapy. Ann Oncol. 2008; 19(11): 1921-6.
48. Maeshima AM, Taniguchi H, Nomoto J, *et al*. Secondary CD5+ diffuse large B-cell lymphoma not associated with transformation of chronic lymphocytic leukemia/small lymphocytic lymphoma (Richter syndrome). Am J Clin Pathol. 2009; 131(3): 339-46.
49. Ogawa S, Yamaguchi M, Oka K, *et al*. CD21S antigen expression in tumour cells of diffuse large B-cell lymphomas is an independent prognostic factor indicating better overall survival. Br J Haematol. 2004; 125(2): 180-6.
50. Went P, Zimpfer A, Tzankov A, Dirnhofer S. CD5 expression in de novo diffuse large B-cell lymphomas. Ann Oncol. 2009; 20(4): 789-90.
51. Linderoth J, Jerkeman M, Cavallin-Stahl E, Kvaloy S, Torlakovic E, Nordic Lymphoma Group S. Immunohistochemical expression of CD23 and CD40 may identify prognostically favorable subgroups of diffuse large B-cell lymphoma: a Nordic Lymphoma Group Study. Clin Cancer Res. 2003; 9(2): 722-8.
52. Arber DA, Orazi A, Hasserjian R, *et al*. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016; 127(20): 2391-405.
53. Perry AM, Alvarado-Bernal Y, Laurini JA, *et al*. MYC and BCL2 protein expression predicts survival in patients with diffuse large B-cell lymphoma treated with rituximab. Br J Haematol. 2014; 165(3): 382-91.
54. Johnson NA, Slack GW, Savage KJ, *et al*. Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol. 2012; 30(28): 3452-9.
55. Hu S, Xu-Monette ZY, Tzankov A, *et al*. MYC/BCL2 protein coexpression contributes to the inferior survival of activated B-cell subtype of diffuse large B-cell lymphoma and demonstrates high-risk gene expression signatures: a report from The International DLBCL Rituximab-CHOP Consortium Program. Blood. 2013; 121(20): 4021-31; quiz 250.
56. Tibiletti MG, Martin V, Bernasconi B, *et al*. BCL2, BCL6, MYC, MALT 1, and BCL10 rearrangements in nodal diffuse large B-cell lymphomas: a multicenter evaluation of a new set of fluorescent in situ hybridization probes and correlation with clinical outcome. Hum Pathol. 2009; 40(5): 645-52.