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Research Paper

Clinical Significance of *BCL2*, C-*MYC*, and *BCL6* Genetic Abnormalities, Epstein-Barr Virus Infection, CD5 Protein Expression, Germinal Center B Cell/Non-Germinal Center B-Cell Subtypes, Co-expression of MYC/BCL2 Proteins and Co-expression of MYC/BCL2/BCL6 Proteins in Diffuse Large B-Cell Lymphoma: A Clinical and Pathological Correlation Study of 120 Patients

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Abstract

Background: Clinical significance of germinal center B-cell (GCB) and non-GCB sub-categorization, expression of MYC, BCL2, BCL6, CD5 proteins and Epstein Barr virus encoded RNA (EBER) positivity in diffuse large B-cell lymphoma (DLBCL) remain controversial. Could these biomarkers accurately identify high risk DLBCL patients? Are MYC, BCL2 and BCL6 proteins expression feasible as baseline testing to predict *c-Myc*, *BCL2* or *BCL6* gene rearrangements?

Aims: To investigate prognostic values of GCB/non-GCB sub-categorization, Double Protein Expression Lymphoma (DPL), Triple Protein Expression Lymphoma (TPL), positivity of CD5 protein and EBER in patients with DLBCL disease. To evaluate correlation between *BCL2* , *c-Myc* and *BCL6* gene rearrangements with BCL2, MYC and BCL6 proteins expression.

Methods: Diagnostic tissue samples of 120 DLBCL patients between January 2012 to December 2013 from four major hospitals in Malaysia were selected. Samples were subjected to immunohistochemical staining, fluorescent in-situ hybridization (FISH) testing, and central pathological review. Pathological data were correlated with clinical characteristics and treatment outcome.

Results: A total of 120 cases were analysed. Mean age of diagnosis was 54.1 years ± 14.6, 64 were males, 56 were females, mean follow up period was 25 months (ranged from 1 to 36 months). Of the 120 cases, 74.2% were non-GCB whereas 25.8% were GCB, 6.7% were EBER positive, 6.7% expressed CD5 protein, 13.3% were DPL and 40% were TPL. The prevalence of c-*Myc*, *BCL2*, *BCL6* gene rearrangements were 5.8%, 5.8%, and 14.2%, respectively; and 1.6% were Double Hit Lymphoma (DHL). EBER positivity, DPL, TPL, c-*Myc* gene rearrangement, *BCL2* gene rearrangement, extra copies of *BCL2* gene and *BCL6* gene rearrangement were associated with shorter median overall survival (P<0.05). IPI score was the significant determinants of median overall survival in DPL and TPL (P<0.05). CD5 protein expression and GCB/non-GCB sub-categorization did not affect treatment outcome (P>0.05). Overall, *c-Myc*, *BCL2* and *BCL6* gene rearrangements showed weak correlation with expression of MYC, BCL2 and BCL6 proteins (P>0.05). Fluorescent *in situ* hybridization is the preferred technique for prediction of treatment outcome in DLBCL patients.

Conclusion: *c-Myc*, *BCL2*, and *BCL6* gene rearrangements, EBER expression, DHL, TPL and IPI score are reliable risk stratification tools. MYC, BCL2 and BCL6 proteins expression are not applicable as baseline biomarkers to predict *c-Myc*, *BCL2*, and *BCL6* gene rearrangements.

Key words: diffuse large B-cell lymphoma, *c-Myc, BCL2* and *BCL6* gene rearrangements, diffuse large B-cell lymphoma with CD5 protein expression, diffuse large B-cell lymphoma with positive EBER expression, non-germinal center B-cell subtype, Asia

Introduction

Diffuse large B-cell lymphoma (DLBCL) appears as one of the malignancies of major public health concern, accounting for 30% to 58% and 25% to 35% of non-Hodgkin lymphomas (NHL) in EU5 (France, Germany, Italy, Spain, United Kingdom) and United States of America, respectively [1]. A study at Queen Elizabeth Hospital in Sabah, Malaysia revealed that approximately 65.1% of NHL cases were DLBCL [2]. This disease is genetically heterogenous, exhibits variations in clinical presentation and results in inconsistent treatment outcomes. The International Prognostic Index (IPI) [3] has been routinely used to stratify risk in DLBCL patients in the current clinical setting. The utilization of several genetic and proteomic testing has enabled disease prognostication and facilitated selection of optimum, individualized risk-adapted therapy. Reports of prospective clinical trials have led to application of various treatment approaches in addition to the existing standard regimen RCHOP-like therapy (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone). Examples of such additional treatments are upfront autologous stem cell transplantation (SCT) for patients with advanced stage disease [4], alternative regimen such as DA-EPOCH-R (dose-adjusted etoposide, cyclophosphamide, doxorubicin, vincristine, prednisone, and rituximab) for patients with positive BCL6 protein expression [5], as well as novel therapeutic agents such as Ibrutinib and Bortezomib specifically to downregulate NF-kB pathway in activated B-cell subtype DLBCL [6] [7].

This study sought to identify parameters associated with inferior overall survival (OS). Both immunohistochemical testing (CD20, CD3, Ki67, Pax5, CD10, BCL6, MUM1, MYC protein, BCL2, CD5, CD23, Cyclin D1 and EBER) and fluorescent in-situ hybridization testing (FISH) (*BCL2* , *BCL6* and *c-Myc* gene rearrangements) were done to correlate the pathological findings with the patients' clinical features and treatment outcomes. Though various biomarkers have been evaluated, the results were controversial [8][9][10], and they were further complicated by introduction of new variants or subtypes of aggressive B cell lymphomas [11]. This study will provide us a guideline on selecting biomarkers to identify high risk DLBCL patients.

Materials and methods

Ethical approval and consent to participate

This research was performed in accordance with the Declaration of Helsinki. Analysis on archival diagnostic biopsy specimens of diffuse large B-cell lymphoma patients was approved by Medical Research Ethics Committee, Ministry of Health Malaysia with research ID NMRR-13-973-17683. Formalin-fixed paraffin embedded tissue samples used in this study were leftover materials from the patients' diagnostic samples. All these samples were anonymized during the study and Malaysian Research Ethics Committee waived the need for written informed consent.

Study Design and study subjects

This was a retrospective cohort study on pathological, clinical features and treatment outcome of DLBCL patients. All de novo DLBCL, not otherwise specified [12] diagnosed at four major public hospitals in Malaysia [Hospital Ampang – National Hematology Referral Centre (Selangor), Queen Elizabeth Hospital (Sabah), Hospital Pulau Pinang (Penang) and Sarawak General Hospital (Kuching, Sarawak)] in year 2012 and 2013 were included in this study. A 3-year retrospective patients' clinical data (from January 2012 till December 2015) was collected. Patients with primary central nervous system lymphoma, primary mediastinal B-cell lymphoma were excluded from this study. Diagnostic formalin-fixed paraffin-embedded tissue blocks for the selected cases were identified by the pathologists and were obtained from the respective laboratories. These samples were sent to Hematopathology Laboratory, Hematology Department of Hospital Ampang for hematoxylin-eosin staining, immunohistochemical staining and FISH testing. The slides produced were reviewed by five pathologists from the four hospitals in two sessions of central pathological review meeting.

Clinical data of these DLBCL patients were retrieved from the hospital information system of these four major public hospitals. Sixty-four patients received RCHOP-like chemotherapy, 34 were treated with CHOP-like chemotherapy, eight were treated with Methotrexate-based regimen, two patients received dose adjusted EPOCH therapy and the remaining six received palliative therapy only. Six patients were given SCT after the first line chemotherapy treatments. For the purpose of homogeneity of treatment for survival analysis, only patients who were uniformly treated with RCHOP-like or CHOP-like chemotherapy without autologous transplantation were included in the statistical analysis.

Treatment outcomes analysed included complete remission (CR) at post 6 cycles of RCHOP-like or CHOP-like chemotherapy treatment, overall survival (OS) and 2-year survival rate. CR was defined as regression of nodal mass to less than 1.5 cm in the longest transverse diameter of a lesion, no extra-lymphatic sites of disease, normalized size of organ

affected, no disease detected in bone marrow, absence of new lesion, and free from non-measured lesions [13]. OS was measured from the date of diagnosis until 31st December 2015 or till death (with death could be attributed to either disease related or treatment related).

Hematoxylin-Eosin and Immunohistochemistry staining

2 μm thick tissue sections were prepared from paraffin tissue blocks, place on charged slides (Matsunami Platinum PRO Adhesive Glass Slide) and were stained with hematoxylin-eosin stains and immunohistochemistry staining. Reactive lymphoid hyperplasia tonsil tissue samples were used as control tissue for CD20, CD3, PAX-5, Ki67, CD5, CD23, Cyclin D1, CD10, BCL6, MUM1, BCL2 antibodies used in this study. As for MYC staining, positive control tissue samples for MYC protein were applied whereas tissue samples positive for Eptein-Barr virus were used as control tissue for Epstein-Barr virus encoded ribonucleic acid (RNA) (EBER) assay.

Cut-off points for CD10, BCL6, MUM 1 and BCL2 immunohistochemical staining

Hans' algorithm [14] was applied to classify DLBCL into germinal center B-cell subtype (GCB) and non-GCB subtype. Cut-off point for CD10 protein was >30% of positive membranous staining on tumor cells; BCL6 protein was >30% positivity of tumor nuclei; MUM1 protein was >30% nuclear positivity on tumor cells. Cut-off points set for MYC was >40% nuclear positivity on tumor nuclei whereas BCL2 protein was >50% of tumor cells with positive cytoplasmic staining reaction, similar to that used in other study [15].

Cut-off point for Epstein-Barr virus-encoded RNA (EBER) *in situ* hybridization testing

In this study, a cut-off point of 50% positivity on tumor cells was applied for EBER positive DLBCL as previously described [16].

Immunohistochemistry Antibodies

Immunohistochemistry staining was performed on Ventana BenchMark GX using OptiView DAB IHC Detection Kit (Ventana Medical Systems, Tucson USA) whereas EBER immunohistochemical staining was performed on Bond-Max instrument (Leica, Newcastle Upon Tyne, UK) using Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle Upon, United Kingdom). The test protocols and the antibodies used for immunohistochemistry staining are shown in Table 1.

Fluorescent *in situ* hybridization testing (FISH)

Fluorescent *in situ* hybridization analysis was performed on 3 μM thick tissue sections to determine genes arrangements of this study cohort. DNA break apart probes used were Dako *MYC* (8q24), Dako *BCL2* (18q21) and Dako *BCL6* (3q27). The positive threshold set for gene rearrangement was more than 10% of the tumor cells demonstrate split signals; with distance between the separated green and red signals twice the size of the biggest signal [17]. In addition, a case was considered positive for extra gene copies if more than 10% of the tumor cells within the tissue specimen expressed three or more pairs of normal fused signals or without gene rearrangement [18].

Statistical analysis

Association between clinicopathological data of study subjects, their immunohistochemistry biomarkers expression, and genetic features were performed using either the Fisher's exact analysis or Pearson Chi Square test. Mann-Whitney test was applied to compare median age of diagnosis between EBER positive and EBER negative group.

The prognostic implications which include CR rate (within 12 months after initiation of treatment), and 2-year survival rate of *c-Myc*, *BCL2* and *BCL6* gene rearrangements, EBER positivity, CD5 protein expression and GCB/non-GCB subtypes were evaluated using the *Fisher's exact* test or *Pearson Chi square* analysis. Correlation between MYC, BCL2 and BCL6 proteins and *c-Myc*, *BCL2* and *BCL6* gene rearrangements were performed by Pearson Bivariate Correlations analysis. OS was measured from the date of diagnosis until patient's death. Median OS was performed using Kaplan-Meier graph, while the comparison of median OS between groups were estimated using the log-rank test. *P*-value <0.05 was considered statistically significant.

Results

Initially, a total of 278 cases were selected and evaluated for this study, 91 cases were then excluded due to incomplete clinical data (67 cases), inadequate tissue biopsy material for testing (60 cases), poorly preserved tissue samples (15 cases) and poor quality FISH signals (16 cases). Eventually, only 120 cases of de novo DLBCL were included in this study.

The age range of the diagnosis of our DLBCL cohort was 18 to 86 years. The mean age of diagnosis was 54.1 ± 14.6, as shown in Table 2, with male : female ratio of 1.14 : 1. From a total of 120 patients, 43.3% of the diagnostic specimens were lymph node tissue biopsy, Waldeyer's ring samples accounted for 14.2% and the remaining 42.5% of samples were from extranodal sites. The most common extranodal sites were gastrointestinal tract (20.9%), followed by head and neck (8.4%), respiratory system (5.0%), skin and

soft tissue (3.3%), skeletal tissue (2.5%), genitourinary (0.8%), breast tissue (0.8%) and pancreas (0.8%).

Using Hans algorithm, 74.2% of 120 patients were classified as non-GCB subtype and only 25.8% as GCB subtype. Both GCB and non-GCB showed comparable CR rates [(RCHOP-like treated-GCB subtype versus RCHOP-like treated non-GCB subtype: 50.0% versus 69.5%, *P=0.142*); (CHOP-like treated-GCB subtype versus CHOP treated non-GCB subtype: 28.5% versus 25.9%, *P=0.872*)] and comparable median OS [(RCHOP-like treated-GCB subtype versus RCHOP-like treated non-GCB subtype: 27.6 months ± 2.9 versus 29.0 months ± 1.7, *P=0.361*, CHOP treated-GCB subtype versus CHOP treated non-GCB subtype: 19.7 months ± 4.4 versus 21.6 months ± 2.3, *P=0.895*)].

No significant correlation was found between DLBCL subtypes with age of diagnosis, gender, IPI scores, disease stage, serum lactate dehydrogenase level (LDH) and 2-year survival rate (*P>0.05*). However, incidence of nodal DLBCL (excluding Waldayer's ring) was higher among GCB subtype, while extranodal DLBCL was more frequent in non-GCB subtype (*P=0.061*).

Epstein Barr virus encoded RNA (EBER) was detected in 8 patients (6.7%). No significant difference in distribution of GCB and non-GCB subtype was found between EBER positive group and EBER negative group (*P=0.424*). Mean age of diagnosis for EBER positive group was not significantly different from EBER negative group (58.0 ± 10.7 versus 53.8 ± 14.9, *P= 0.573*). Majority of the EBER positive cases had low IPI scores (1 to 2), (87.5%, *P=0.137*). Of the 8 EBER positive cases, one patient was treated with SCT, one patients with palliative therapy; statistical analysis were performed on five patients who were treated with RCHOP-like chemotherapy and one patient on CHOP-like therapy. RCHOP-like treated-EBER positive group had significantly shorter OS period than the RHOP-like treated-EBER negative group (17.8 months ± 4.3 versus 29.5 months ± 1.5, *P =0.008*) and lower 2-year survival rate (20% versus 76%, *P=0.008*). Within EBER positive group, disease stage was the key factor affecting OS. EBER positive patients with stage lll to lV disease had significant shorter OS duration compared to the EBER positive patients with stage l to ll disease [7.5 months ± 2.5 versus 24.6 months ± 3.5, *P=0.039*]. Patient's age, gender, site of disease and GCB/non-GCB subtypes showed no correlation with overall survival.

CD5 protein expression was positive in 8 patients (6.7%) with equal distribution between male and female DLBCL patients (4:4). No significant difference was found between mean age of diagnosis for patients with positive CD5 protein expression (58.3 years ± 7.8) and those with negative CD5 protein expression (53.8 years ± 15.0) with *P=0.542*. Positive CD5 protein expression is associated with aggressive disease and poor prognosis. Majority of the patients with positive CD5 protein expression had high IPI score (more than 2) (75% versus 39.3%, *P=0.066*) and advance stage disease (stage III to IV, 87.5% versus 54.5%, *P=0.069*). From a total of 8 patients with CD5 protein expression, 4/8 were treated with CHOP chemotherapy, 1/8 with RCHOP-like chemotherapy, 2/8 with palliative therapy and 1/8 were treated with methotrexate-based chemotherapy. In RCHOP-like treatment group, no significant difference was observed in median OS between CD5 protein positive group and CD5 protein negative group (9 months ± 0 versus 24.1 months ± 1.3, *P=0.732*). For CHOP-treated group, the median OS of CD5 protein positive group was also statistically insignificant from CD5 protein negative group (12.5 months ± 5.5 versus 22.3 months ± 2.1, *P=0.257*). CD5 protein positive-high IPI scores patients who were treated with CHOP-like had the worst treatment outcome, their survival period ranged from 2 months to 14 months (median survival period of 6.6 months). 2-year OS rate for CHOP-like treated-CD5 protein positive group was lower than the CHOP-like treated-CD5 protein negative group (25% versus 46%, *P=0.257*).

The prevalence of Double Protein Expression Lymphoma (DPL) (co-expression of MYC and BCL2 protein), and Triple Protein Expression Lymphoma (TPL) (co-expression of MYC, BCL2 and BCL6 proteins) in our cohort were 13.3% (16 patients) and 40% (48 patients), respectively. Their clinicopathological characteristics and treatment outcomes are shown in Table 3.

The prognostic values of patients with positive MYC/BCL2 proteins co-expression (DPL) were evaluated. DPL was more prevalent among older patients (60.3 years ± 15.8 versus 52.1 years ±14.7; *P=0.048*). All of them were non-GCB subtype (*P=0.008*) and this group had higher rate of MYC gene rearrangement (18.7%) (*P=0.02*). Shorter median OS was observed among RCHOP-like treated-DPL compared to RCHOP-like treated-non-DPL (17.7 months ± 4.4 versus 29.8 months ± 1.9, *P=0.080*).

As for those on CHOP-like treatment, shorter median OS was also observed in DPL group compared to the non-DPL group (19.6 months ± 4.6 versus 27.6 months ± 2.6, *P=0.089*).

Within the DPL group, IPI score was still a significant factor in determining overall survival period. Median OS of RCHOP-like treated-DPL patients with high IPI score of 3 to 5 was significantly shorter than those with low IPI score of 1 to 2 (13.5 months ± 10.7 versus 32.5 months ± 9.9, *P=0.018*). Such observation was not found in CHOP-like treated

group. Median OS of CHOP-like treated-DPL patients with high IPI score of 3 to 5 was not statistically different from those with low IPI score of 1 to 2 (17.5 months ± 10.0 versus 25.0 months ± 9.2; *P=0.895*).

Approximate 40% of the patients were TPL. Median OS of RCHOP-like treated-TPL group was shorter compared to the RCHOP-like treated-non-TPL group (22.6 months ± 1.9 versus 29.8 months ± 1.9; *P=0.053*). Similar finding was found in patients treated with CHOP-like therapy. Median OS of CHOP-like treated-TPL group was significantly shorter compared to CHOP-like treated-non-TPL group (14.1 months ± 3.1 versus 27.6 months ± 2.6, *P=0.002*).

Table 1. Antibodies and protocols used for immunohistochemistry staining

	Antibody	Clone	Monoclonal/ polyclonal	Manufacturer	Epitope Retrieval Condition	Antibody Incubation Period
1	CONFIRM Anti-CD20	L26	Mouse Monoclonal	Ventana Medical System, Tucson, United States	CC1 16 min	10 min
2	Anti-BCL-2	SP66	Rabbit monoclonal		CC1 64 min	16 min
3	Anti-CD5	SP19			CC1 32 min	16 min
4	Anti-CD23	SP23			CC1 48 min	16 min
5	Anti-Ki-67	30-9			CC1 64 min	16 min
6	MUM1	MRQ-43			CC1 32 min	16 min
7	Anti-PAX5	SP34			CC1 32 min	16 min
8	Anti-MYC	Y69			CC1 64 min	32 min
9	Anti-human BCL6 Protein	PG-B6p	Mouse Monoclonal	Dako, Glostrup, Denmark	CC1 32 min	52 min
10	Anti-human CD10	56C6			CC1 24 min	32 min
11	Anti-human CD3		Polyclonal rabbit		CC1 32 min	16 min
12	Anti-human Cyclin D1	EP12	Monoclonal rabbit		CC1 32 min	32 min
13	ISH EBER probe			Leica Biosystems, Newcastle Upon, United Kingdom)		

Table 2. Clinicopathological characteristics and treatment outcomes of diffuse large B-cell lymphoma subtypes based on cell of origin, EBER Positive diffuse large B-cell lymphoma and CD5 protein expression.

Clinical and pathological characteristics	Overall	Diffuse large B-cell lymphoma subtypes			EBER			CD5 Protein Expression		
		GCB	Non-GCB	P value	Positive	Negative	P value	Positive	Negative	P value
Mean age, years (SD)	54.1 (14.6)	52.4 (13.5)	54.7 ± 15.1	0.286	58.0 (10.7)	53.8 (14.9)	0.573	58.3 (7.8)	53.8 (15.0)	0.542
Age > 60 years	45/120 (37.5%)	11/31 (35.5%)	34/89 (38.2%)	0.788	4/8 (50.0%)	41/112 (36.6%)	0.471	3/8 (37.5%)	42/112 (37.5%)	0.655
Gender - Male	64/120 (53.3%)	13/31 (41.9%)	51/89 (57.3%)	0.140	5/8 (62.5%)	59/112 (52.6%)	0.722	4/8 (50.0%)	60/112 (53.6%)	0.564
Diagnostic Specimen sites										
Lymph nodes	52/120 (43.3%)	20 /31 (64.5%)	32/89 (36.0%)	**0.061**	4/8 (50.0%)	48/112 (42.8%)	0.942	4/8 (50.0%)	48/112 (42.9%)	0.103
Waldeyer's ring	17/120 (14.2%)	1/31 (3.2%)	16/89 (18.0%)		1/8 (12.5%)	16/112 (14.3%)		1/8 (12.5%)	16/112 (14.2%)	
Extranodal sites	51/120 (42.5%)	10/31 (32.3%)	41/89 (46.0%)		3/8 (37.5%)	48/112 (42.9%)		3/8 (37.5%)	48/112 (42.9%)	
Subtype										
GCB	31/120 (25.8%)	NA	NA	NA	3/8 (37.5%)	28/112 (25.0%)	0.424	0/8 (0%)	31/112 (27.7%)	0.111
Non-GCB	89/120 (74.2%)	NA	NA	NA	5/8 (62.5%)	84/112 (75.0%)		8/8 (100.0%)	81/112 (72.3%)	
CD5 positive	8/120 (6.7%)	0/31 (0%)	8/89 (9.0%)	0.111	0/8 (0%)	8/112 (7.1%)	0.566	NA	NA	NA
EBER positive	8/120 (6.7%)	3/31 (9.7%)	5/89 (5.6%)	0.424	NA	NA	NA	0/8 (0%)	8/112 (7.1%)	0.566
***c-Myc* gene rearrangement**										
Positive	7/120 (5.8%)	3/31 (9.6%)	4/89 (3.3%)	0.536	0/8 (0%)	7/112 (6.2%)	0.736	0/8 (0%)	6/112 (5.4%)	0.767
Extra copies	1/120 (0.8%)	0/31 (0%)	1/89 (1.1%)		0/8 (0%)	1/112 (/0.9%)		0/8 (0%)	1/112 (0.9%)	
***BCL2* gene rearrangement**										
Positive	7/120 (5.8%)	3/31 (9.7%)	4/89 (4.4%)	0.198	1/8 (12.5%)	6/112 (5.3%)	0.612	0/8 (0%)	8/112 (7.1%)	0.101
Extra copies *BCL2 gene*	6/120 (5.0%)	0/31 (0%)	6/89 (6.7%)		0/8 (0%)	6/112 (5.3%)		2/8 (25%)	5 /112 (4.5%)	
***BCL6* gene rearrangement**										
Positive	17/120 (14.2%)	5/31 (16.1%)	12/89 (13.4%)	0.767	1/8 (12.5%)	16/112 (14.2%)	0.684	1/8 (12.5%)	15/112 (13.4%)	0.711
IPI score >2	50/120 (41.7%)	11/31 (35.5%)	39/89 (43.8%)	0.417	1/8 (12.5%)	49/112 (43.8%)	0.137	6/8 (75.0%)	44/112 (39.3%)	**0.066**
LDH– Raised	89/120 (74.2%)	26/31 (83.9%)	63/89 (70.8%)	0.176	7/8 (87.5%)	82/112 (73.2%)	0.678	7/8 (87.5%)	82/112 (73.2%)	0.678
Stage– III, IV	68/120 (56.7%)	16/31 (51.6%)	52/89 (58.4%)	0.510	4/8 (50.0%)	64/112 (57.1%)	0.726	7/8 (87.5%)	61/112 (54.5%)	**0.069**
Treatment outcome – CR rate										
RCHOP-like	41/64 (64.1%)	9/18 (50.0%)	32/46 (69.5%)	0.142	2/5 (40.0%)	39/59 (66.1%)	0.341	0/1 (0%)	41/63 (65.1%)	0.359
CHOP-like	9/34 (26.5%)	2/7 (28.5%)	7/27 (25.9%)	0.872	0/1 (0%)	9/33 (27.2%)	**0.029**	1/4 (25.0%)	8/30 (26.7%)	0.928
Treatment Outcome – 2-year survival rate										
RCHOP-like	47/64 (73%)	12/18 (66%)	35/46 (76%)	0.361	1/5 (20%)	45/59 (76%)	0.008	0/1 (0%)	46/63 (73%)	0.732
CHOP-like	15/34 (44%)	2/7 (28%)	12/27 (45%)	0.895	0/1 (0%)	15/33 (45%)	**<0.01**	1/4 (25%)	14/30 (46%)	0.257
Treatment Outcome – median OS (IQR) (month)										
RCHOP-like	28.6 (20)	27.6 (21)	29.0 (19.0)	0.361	17.8 (22)	29.5 (19)	**0.008**	9.0 (NA)	24.1 (20.0)	0.732
CHOP-like	21.3 (16)	19.7 (24)	21.6 (15)	0.895	3.0 (NA)	21.8 (15)	**<0.01**	12.5 (24)	22.3 (15)	0.257

CR: complete response; EBER: Epstein-Barr virus encoded ribonucleic acid; GCB: germinal center B-cell; IQR: interquartile range; LDH: lactate dehydrogenase; NA: not available; OS: overall survival; SD: standard deviation

Table 3. Clinicopathological characteristics and treatment outcomes of Double Protein Expression Lymphoma (DPL) and Triple Protein Expression Lymphoma (TPL).

Clinical and pathological characteristics	DPL (MYC+/BCL2+)			TPL (MYC+/BCL2+/BCL6+)		
	Positive	Negative	P value	Positive	Negative	P value
Mean age, years (SD)	60.3 (15.8)	52.1 (14.7)	**0.048**	54.1 (14.9)	53.9 (13.4)	0.810
Age >60 years	9/16 (56.2%)	17/56 (30.3%)	**0.057**	19/48 (39.5%)	17/56 (30.3%)	0.324
Gender – Male	7/16 (43.7%)	27/56 (48.2%)	0.752	30/48 (62.5%)	27/56 (48.2%)	0.144
Diagnostic specimen sites						
Lymph nodes	8/16 (50.0%)	23/56 (41.1%)	0.288	21/48 (43.8%)	23/56 (41.1%)	0.165
Waldeyer's ring	1/16 (6.3%)	6/56 (10.7%)		10/48 (20.8%)	6/56 (10.7%)	
Extranodal sites	7/16 (43.7%)	27/56 (48.2%)		17/48 (35.4%)	27/56 (48.2%)	
Subtype						
GCB	0/16 (0%)	19/56 (33.9%)	**0.008**	12/48 (25.0%)	19/56 (33.9%)	0.392
Non-GCB	16/16 (100%)	37/56 (66.1%)		36/48 (75.0%)	37/56 (66.1%)	
CD5 positive	2/16 (12.5%)	1/56 (1.7%)	0.122	5/48 (10.4%)	1/56 (1.8%)	0.093
EBER positive	1/16 (6.25%)	3/56 (5.3%)	0.643	4/48 (8.3%)	3/56 (5.4%)	0.701
***c-Myc* gene rearrangement**						
Positive	3/16 (18.7%)	2/56 (3.5%)	**0.020**	2/48 (4.2%)	2/56 (3.6%)	0.631
Extra copies	1/16 (6.2%)	0/56 (0%)		0/48 (0%)	0/56 (0%)	
***BCL2* gene rearrangement**						
Positive	1/16 (6.2%)	4/56 (7.1%)	0.736	2/48 (4.2%)	4/56 (7.1%)	0.530
Extra copies	0/16 (0%)	2/56 (3.5%)		4/48 (8.3%)	2/56 (3.6%)	
***BCL6* gene rearrangement**						
Positive	2/16 (12.5%)	8/56 (14.2%)	0.610	7/48 (14.5%)	8/56 (14.3%)	0.591
IPI > 2	9/16 (56.2%)	19/56 (33.9%)	0.106	22/48 (45.8%)	19/56 (33.9%)	0.234
LDH – Raised	11/16 (68.7%)	41/56 (73.2%)	0.635	37/48 (77.1%)	41/56 (73.2%)	0.821
Stage – III, IV	10/16 (62.5%)	29/56 (51.7%)	0.448	29/48 (60.4%)	29/56 (51.8%)	0.431
Treatment outcome – CR rate						
RCHOP-like	2/6 (33.3%)	19/30 (63.3%)	0.210	20/28 (71.4%)	19/30 (63.3%)	0.512
CHOP-like	3/6 (50.0%)	5/16 (31.2%)	0.732	1/12 (8.3%)	5/16 (31.2%)	0.254
Treatment outcome – 2-year survival rate						
RCHOP-like	3/6 (50%)	22/30 (73%)	**0.080**	22/28 (78%)	22/30 (73%)	**0.053**
CHOP-like	3/6 (50%)	10/16 (62%)	**0.089**	2/12 (16%)	11/16 (68%)	**0.002**
Treatment Outcome – median OS (IQR) (month)						
RCHOP-like	17.7 (20)	29.8 (15)	**0.080**	22.6 (17)	29.8 (15)	**0.053**
CHOP-like	19.6 (19)	27.6 (13)	**0.089**	14.1 (16)	27.6 (13)	**0.002**

CR: complete response; EBER: Epstein-Barr virus encoded ribonucleic acid; GCB: germinal center B-cell; IQR: interquartile range; LDH: lactate dehydrogenase; OS: overall survival; SD: standard deviation

IPI score was an important determinant for OS in RCHOP-like treated-TPL group. Patients with high IPI score of 3 to 5 had significant shorter median OS period than the patients with low IPI score of 1 to 2 (23.0 months ± 4.3 versus 33.2 months ± 1.9; *P=0.030*). However, in CHOP-like treated group, median OS of CHOP-like treated-TPL patients with high IPI score was not significantly different from CHOP-like treated-TPL patients with low IPI score (12.6 months ± 3.4 versus 16.6 months ± 4.7; *P=0.602*).

2-year survival rate for RCHOP-like treated-DPL was lower compared to RCHOP-like treated-TPL group (50% versus 78%). 2-year survival rate for CHOP-like treated group was worse compared to the RCHOP-like treated group. 2-year survival rates for CHOP-like treated-DPL group and CHOP-like treated-TPL group were 50% and 16%, respectively.

The prevalence of *c-Myc*, *BCL2,* and *BCL6* gene rearrangements were 7 (5.8%), 7 (5.8%), and 17 (14.1%), respectively. There were two cases of DHL (concurrent *c-Myc* and *BCL2* gene rearrangements), but no Triple Hit Lymphoma (THL) (concurrent *c-Myc*, *BCL2* and *BCL6* gene rearrangements) in our study cohort. The clinicopathological characteristics and treatment outcomes of DLBCL patients with these three gene rearrangements are as shown in Table 4.

c-Myc gene rearrangements were detected in 7 of 120 cases (5.8%). 4.2% had sole *c-Myc* gene rearrangement and 1.6% demonstrated concurrent *c-Myc* and *BCL2* gene rearrangements (DHL). In addition, there was one case of *MYC* gene extra copies.

c-Myc gene rearrangement is an indicator of poor prognosis on both low or high IPI scores and at all disease stages (57.1% with low disease stage of 1 to 2; 57.1% with IPI score of 1 to 2). All *c-Myc* gene rearrangement positive cases (7 of 7) in both RCHOP-like treatment and CHOP-like treatment experienced disease relapse or refractory disease (RCHOP-like treated group 100%, *P=0.014* and CHOP-like treated group: 100%, *P=0.=615*). *c-Myc* gene rearrangement positive group demonstrated rather low rate of 2-year survival rate, 0% in CHOP-like treated group and 33% in RCHOP-like treated group. In contrast, CHOP-like treated *c-Myc* gene rearrangement negative group and RCHOP-like treated *c-Myc* gene rearrangement negative group had

much higher rates of 2-year survival, 50% and 77% respectively. Compared to RCHOP-like treated *c-Myc* gene rearrangement negative group, RCHOP-like treated *c-Myc* gene rearrangement positive group had significant shorter median overall survival period (13.6 months ± 4.6 versus 29.6 months ± 1.4, *P<0.01*). CHOP-like treated-*c-Myc* gene rearrangement positive group also demonstrated significant shorter median overall survival period than the CHOP-like treated *c-Myc* gene rearrangement negative group (6.5 months ± 2.5 versus 22.2 ± 2.0, *P<0.01).*

Table 4. Clinicopathological characteristics of *c-Myc*, *BCL2* and *BCL6* gene rearrangements; association with treatment outcomes and correlation with MYC, BCL2 and BCL6 protein expression

Clinical and Pathological Characteristics	*c-Myc* gene rearrangement				*BCL2* gene rearrangement				*BCL6* gene rearrangement		
	Positive	Negative	Extra copies	P Value	Positive	Negative	Extra copies	P Value	Positive	Negative	P Value
Mean age, year (SD)	58.5 (20.4)	53.6 (14.2)	72 (NA)	0.053	61 (10.5)	53.7 (14.7)	53.1 (16.4)	0.718	54.8 (10.4)	53.9 (15.2)	**0.718**
Age >60 years	3/7 (42.8%)	41/112 (36.6%)	1/1 (100%)	0.531	4/7 (57.1%)	39/107 (36.4%)	2/6 (33.3%)	0.536	6/17 (35.2%)	39/103 (37.8%)	0.839
Gender - Male	3/7 (42.8%)	60/112 (53.5%)	1/1 (100%)	0.843	3/7 (42.8%)	56/107 (52.3%)	5/6 (83.3%)	0.298	5/17 (29.5%)	59/103 (57.2%)	**0.033**
Diagnostic specimen sites											
Lymph node	3 (42.9%)	48 (42.9%)	1 (100%)		6 (85.7%)	44 (41.1%)	2(33.3%)		7 (41.2%)	45 (43.7%)	
Waldeyer's ring	3 (42.9%)	14 (12.5%)	0 (%)	0.503	0 (0%)	15 (14.0%)	2 (33.3%)	0.569	1 (5.9%)	16 (15.5%)	**0.029**
Extranodal sites	1 (14.2%)	50 (44.6%)	0 (0%)		1 (14.3%)	48 (44.9%)	2 (33.3%)		9 (52.9%)	42 (40.8%)	
Subtype											
GCB	3/7 (42.9%)	28/112(25.0%)	0	0.536	3/7 (42.9%)	28/107 (26.2%)	0/6 (0%)	0.198	5/17 (29.4%)	26/103 (25.2%)	0.767
Non-GCB	4/7 (57.1%)	84/112(75.0%)	1		4/7 (57.1%)	79/107 (73.8%)	6/6 (100%)		12/17 (70.6%)	77/103 (74.8%)	
CD5 positive	0/7 (0%)	8/112 (7.1%)	0/1 (0%)	0.736	0/7 (0%)	6/107 (5.6%)	2/6 (33.3%)	0.073	1/17 (5.8%)	7/103 (6.7%)	0.684
EBER positive	0/7 (0%)	8/112 (7.1%)	0/1 (0%)	0.736	1/7 (14.3%)	7/107 (6.5%)	0/6 (0%)	0.612	1/17 (5.8%)	7/103 (6.7%)	0.684
c-Myc gene rearrangement											
Positive	NA	NA	NA	NA	2/7 (28.5%)	5/107 4.7%)	0/6 (0%)	0.191	0/17 (0%)	7/103 (6.7%)	0.331
Extra copies	NA	NA	NA	NA	0/7 (0%)	1/107 (0.9%)	0/6 (0%)		0/17 (0%)	1/103 (0.9%)	
***BCL2* gene rearrangement**											
Positive	2/7 (28.5%)	5/112 (4.4%)	0/1 (0%)	0.191	NA	NA	NA	NA	2/17(11.7%)	5/103 (4.8%)	0.229
Extra copies	0/7 (0%)	6/112 (5.3%)	0/1 (0%)		NA	NA	NA	NA	1/17 (5.8%)	5/103 (4.8%)	
***BCL6* gene rearrangement**											
Positive	0/7 (0%)	17/112 (15.1%)	0/1 (0%)	0.649	2/7 (28.5%)	14/107 (13.0%)	1/6 (16.6%)	0.331	NA	NA	NA
IPI > 2	3/7 (42.9%)	47/112 (41.9%)	0/1 (0%)	0.697	3/7 (42.8%)	43/107 (40.2%)	4/6 (66.6%)	0.491	9/17 (52.9%)	41/103(39.8%)	0.309
LDH - Raised	7/7 (100%)	82/112 (73.2%)	0/1 (0%)	0.057	7/7 (100%)	77/107 (71.9%)	5/6 (83.3%)	0.313	13/17 (76.4%)	76/103 (73.7%)	0.565
Stage - III, IV	3/7 (42.9%)	65/112 (58.4%)	0/1 (0%)	0.334	6/7 (85.7%)	58/107 (54.2%)	4/6 (66.7%)	0.211	11/17 (64.7%)	57/103 (55.3%)	0.470
Treatment outcome – CR rate											
RCHOP-like	0/3 (0.0%)	41/60 (68.3%)	0/1 (0%)	**0.014**	NA	40/60 (66.6%)	1/4 (25%)	0.093	2/7 (28.5%)	39/57 (68.4%)	**0.038**
CHOP-like	0/2 (0.0%)	9/32 (28.1%)	NA	0.615	0/3 (0%)	9/31 (34.7%)	NA	**0.004**	1/3 (33.3%)	8/31 (25.8%)	0.720
Treatment outcome – 2-year survival rate											
RCHOP-like	1/3 (33%)	46/60 (77%)	0/1 (0%)	<0.01	NA	47/60 (78%)	0/4 (0%)	<0.01	3/7 (42%)	44/57 (77%)	0.018
CHOP-like	0/2 (0%)	16/32 (50%)	NA	<0.01	0/3 (0%)	15/31 (48%)	NA	<0.01	1/3 (33%)	14/31 (45%)	0.619
Treatment outcome – median OS (IQR) (month)											
RCHOP-like	13.6 (NA)	29.6 (19)	1.5	**<0.01**	NA	30.0 (19)	8.2 (16)	**<0.01**	16.6 (7)	29.6 (17)	**0.018**
CHOP-like	6.5 (5)	22.2 (21)	NA	**<0.01**	7.0 (11)	22.6 (21)	NA	**<0.01**	15.3 (6)	21.7 (14)	0.619
Correlation with protein expression											
Positive MYC protein expression	7/7 (100%)	70/112(62.5%)	1/ 1 (100%)	0.066	NA	NA	NA	NA	NA	NA	NA
Positive BCL2 protein expression	NA	NA	NA	NA	6/7 (85.7%)	86/107 (80.3%)	6/6 (100%)	0.841	NA	NA	NA
Positive BCL6 protein expression	NA	NA	NA	NA	NA	NA	NA	NA	11/17 (64.7%)	76/103 (73.7%)	0.558

CR: complete response; EBER: Epstein-Barr virus encoded ribonucleic acid; GCB: germinal center B-cell; IPI: International Prognostic Index; IQR: interquartile range; LDH: lactate dehydrogenase; NA: not applicable; OS: overall survival; SD: standard deviation

The only case with extra copies of *c-Myc* gene was a 71 year-old male patient who had DLBCL on his lymph node. His tumor was categorized as non-GCB subtype, stage ll disease, IPI score of 1, and achieved partial remission after RCHOP-like therapy. This patient survived for only 1.5 month after diagnosis, and died due to sepsis.

Of the 120 cases analyzed, 7 cases were positive for *BCL2* gene rearrangement (5.8%) and 6 cases were found to have extra copies of *BCL2* gene. The

prevalence of *BCL2* gene rearrangement was higher among GCB subtype-DLBCL (9.7%) compared to the non-GCB subtype-DLBCL (4.5%). Of the 7 cases, 4 underwent SCT after receiving RCHOP-like chemotherapy regimens, and the remaining 3 patients were treated with CHOP-like chemotherapy; none of these three patients achieved complete remission (*P=0.004*). Significant shorter median overall survival period was observed in CHOP-like treated-*BCL2* gene rearrangement positive group compared to CHOP-like treated-*BCL2* gene rearrangement negative group (7.0 months ± 3.5 versus 22.6 months ± 2.0, *P<0.01*).

2-year survival rate for CHOP-like treated-*BCL2* gene rearrangement positive group was 0%; and 48% for CHOP-like treated-*BCL2* gene rearrangement negative group. No significant correlation was observed between *BCL2* gene rearrangement and IPI score (42.8% with high IPI scores of 3 to 5, *P=0.491*); and with disease stage (85.7% with high disease stage lll to lV, *P=0.211*).

Of the 6 cases of extra copies of *BCL2* gene, four patients were treated with RCHOP-like chemotherapy, one was treated with Methotrexate based chemotherapy, and the remaining one patient was not fit for treatment. Extra copies of *BCL2* gene were associated with lower CR rate (25% versus 66.6%, *P=0.093*). Overall, probability of survival at 24 months was 0% for extra copies of *BCL2* gene group and 78% for those without extra copies of *BCL2* gene. Median OS for RCHOP treated patients with extra copies of *BCL2* gene was significantly shorter than those without extra copies of *BCL2* gene (8.2 months ± 4.9 versus 30.0 ± 1.4, *P<0.01*). Extra copies of *BCL2* gene were often found in non-GCB subtype (6 of 6 cases or 100%, *P=0.198*), had elevation of serum LDH (5 of 6 cases or 83.3%, *P=0.313*) and expressed BCL2 protein (6 of 6 cases or 100%, *P=0.841*).

BCL6 genetic abnormalities were detected in 14.1% (17 cases); 11.6% (14 of 120) of them had sole *BCL6* gene rearrangement, 1.6% (two cases) had concurrent *BCL2* and *BCL6* gene rearrangements and 1 case (0.8%) of concurrent *BCL6* gene rearrangement and extra copies of *BCL2* gene. *BCL6* gene rearrangement was more prevalent among female patients (70.5%, *P=0.033*) and majority had extranodal disease involvement (52.9%, *P=0.029*). Of the 17 patients with *BCL6* gene rearrangement, seven patients were treated with RCHOP-like chemotherapy, three patients with CHOP chemotherapy, three patients with SCT, two patients with EPOCH, one patient with Methotrexate regimen, and the remaining one patient with palliative therapy. In RCHOP-like treated group, CR rate was much higher in of *BCL6* gene rearrangement negative patients compared to the *BCL6* gene rearrangement positive patients (68.4% versus 28.5%, *P=0.038*). RCHOP-like treated *BCL6* gene rearrangement positive group had shorter median OS compared to the RCHOP-like treated *BCL6* gene rearrangement negative group (16.6 months ± 3.5 versus 29.6 months ± 1.5, *P=0.018*). 2-year survival rate for RCHOP-like treated-*BCL6* gene rearrangement positive group was rather low compared to RCHOP-like treated-*BCL6* gene rearrangement negative group (42% versus 77%).

In this study, weak correlation was found between MYC protein expression and *c-Myc* gene rearrangements (*P=0.066*). No significant correlation was found between BCL2 protein expression and *BCL2* gene rearrangement (*P=0.841*); and between BCL6 protein expression and *BCL6* gene rearrangement (*P=0.558*).

Comparing the prognosis values of these three gene rearrangements, we found no significant difference in the median OS between RCHOP-like treated-*c-Myc* gene rearrangement positive group and RCHOP treated-*BCL6* gene rearrangement positive group (13.6 months ± 4.6 for versus 16.6 months ± 3.5, *P=0.837)*; with 2-year survival rate for RCHOP treated-*cMyc* rearrangement positive group versus RCHOP treated-*BCL6* gene rearrangement positive group of 33% versus 42%.

None of the CHOP-like treated patients with positive *c-Myc* or *BCL2* gene rearrangements survived for 24 months while 33% of the patients with *BCL6* gene rearrangement survived for more than 24 months. The differences in median OS for these three groups with positive *c-Myc*, *BCL2* and *BCL6* gene rearrangements were statistically insignificant (6.5 months ± 2.5 for positive *c-Myc* gene rearrangement group, 7.0 months ± 3.5 for *BCL2* gene rearrangement positive group, and 15.3 months ± 6.1 for *BCL6* gene rearrangement positive group, *P=0.191*) .

Discussion

The clinicopathological features of our study cohort were quite distinct compared to those reported in western countries. Median age of diagnosis for this study cohort was 54.1 years ± 14.6; it was 70 years for the western DLBCL population [19]. Male gender has been found to be associated with poorer treatment outcome [20]. The male to female ratio for this study cohort (1.14:1) was similar to the Japanese's (1.18:1), but was lower than the Caucasian's (1.7:1) [21]. The diagnostic specimen sites for this study cohort (43.3% of lymph nodes, 14.2% of Waldeyer's ring samples, 42.5% of extranodal tissue) were consistent with the western's with 60% of nodal disease and 40% of extranodal involvement [22].

Incidence rate of EBER positive cases was quite low (6.7%) and majority of them had low IPI scores of

1 to 2 (87.5%), which was shown in Nicolae A et al. 2015's study [23]. EBER positivity showed no correlation with older age of diagnosis and sites of disease. These findings contradict previous publications which suggested EBER positivity is associated with old age [24] and was frequent in extranodal [25]. Despite low IPI scores, EBER-positive group demonstrated poorer treatment outcome compared to the EBER-negative group, indicating that EBER positivity is an independent risk factor of poor prognosis. RCHOP-like treatment did not improve treatment outcome of EBER positive patients, as evident in previous publications [25][26].

Clinicopathological characteristics of Malaysian DLBCL with positive CD5 protein expression diverge from the western population and Japanese who demonstrated older median age diagnosis (63 years), female preponderance, and predominant of extranodal involvement [27][28]. However, all studies demonstrated similar treatment failure pattern. In this study cohort, disease refractory rate was rather high (75%) among patients with positive CD5 protein expression. Similarly, Thakral et al. 2017 and Miyazaki et al. 2011 reported higher disease relapse in the central nervous system of diffuse large B-cell lymphoma patients with positive CD5 protein expression [29][30], while Alinari L et al. 2016 reported 71% of disease relapse in positive CD5 protein expression-DLBCL patients treated with SCT [31]. The insignificant difference in median OS period between patients with and without positive CD5 protein expression could be due to small sample size in this study cohort.

The prevalence of GCB (25.8%) and non-GCB (74.2%) in our cohort is comparable to other Asian countries (GCB 29%, non-GCB 71%) [32] and another study in Malaysia study [33], but the westerners reported higher percentage of GCB subtype (42%) [14]. In this study, no significant difference in treatment outcome was found between GCB and non-GCB subtypes ($P>0.05$). Disease prognostication based on GCB/non-GCB sub-categorization in previous publications was contradictory. Some studies suggested that GCB subtype patients have superior treatment outcome than the non-GCB subtype [34][35][36], whereas a few studies showed no significant difference in OS and disease free survival period between GCB subtype and non-GCB subtype [33][37]. Our results showed that there was no significant difference in CR rate ($P=0.142$) and median OS ($P=0.361$) between GCB subtype and non-GCB subtype in RCHOP-like treated-DLBCL patients, which is in concordance with some published literatures [33][38][39][40]. Likewise for patients on CHOP-like treatment (CR rate $P=0.872$, median OS $P=0.895$).

Our results showed that DPL had poorer median OS for both RCHOP-like and CHOP-like groups. The insignificant *P value* of $P=0.080$ and $P=0.089$ in our results were most probably due to small sample size. International prognostic index remains as a significant factor affecting OS of the DPL patients. Other study using dual immunohistochemistry technique with cutoff value of 0.12% for positive MYC/BCL2 proteins co-expression also showed consistent findings [41]. In this study, comparison of OS between DPL and DHL cannot be performed as both the DHL patients had been treated with SCT.

Besides that, TPL patients had significant poorer median OS compared to the non-TPL, and the finding is consistent with another study which showed that TPL is associated with inferior OS and worse progression free survival [42].

The prevalence of *c-Myc* (5.8%), *BCL2* (5.8%) and *BCL6* (14.1%) gene rearrangements in our study cohort were lower compared to the western countries, with *c-Myc* of 7% [43], *BCL2* of 18.3% [44] and *BCL6* of 19.5% [45]. All three *c-Myc*, *BCL2*, *BCL6* gene rearrangements and extra copies of *BCL2* gene were independent prognostic factors for inferior OS ($P<0.05$) and low CR rates. In RCHOP-like-treated group, disease relapse or refractory rates were higher among patients with *c-Myc* gene rearrangement (100%, $P=0.014$); extra copies of *BCL2 gene* (75%, $P=0.093$) and also *BCL6* gene rearrangement (71.5%, $P=0.038$). RCHOP-like and CHOP-like chemotherapy regimens did not improve treatment outcomes of patients with *c-Myc*, *BCL2* and *BCL6* gene rearrangements and extra copies of *BCL2* gene. It has been suggested that deregulated *c-Myc* gene could activate γH2AX foci and sensitizes cellular DNA repair machinery and contribute to chemoresistance [46]. In addition, changes to *BCL2* gene copy number or *BCL2* gene structure have also been identified as the mechanisms contributing to treatment resistance [47].

The incidence of DHL is rather uncommon (1.6%) in our Malaysian DLBCL study cohort. This is most likely due to younger DLBCL patients in Malaysia (median age of diagnosis of 54.1 years ± 14.6) compared to the western countries (with median age of diagnosis of 70.6 years) [48].

Conclusion

EBER positivity, *c-Myc*, *BCL2*, *BCL6* gene rearrangements or extra copies of these genes, IPI score, DPL and TPL are useful prognostication tools in DLBCL. No significant correlation were found between treatment outcomes and GCB/non-GCB sub-categorization; and expression of CD5 protein. Immunohistochemical staining of MYC, BCL2 and BCL6 proteins cannot be used as baseline markers to

predict *c-Myc* , *BCL2* , *BCL6* gene rearrangements.

Abbreviations

EBV: Epstein-Barr virus; DA-EPOCH-R: dose-adjusted etoposide, cyclophosphamide, doxorubicin, vincristine, prednisone, and rituximab; EBER: Epstein Barr virus encoded RNA; RCHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; IPI: international prognostic index score; BCL2: b-cell lymphoma-2; MYC: Myelocytomatosis; BCL6: b-cell lymphoma-6; CD10: cluster of differentiation 10; MUM1: multiple myeloma-1; CD5: cluster of differentiation-5; DPL: Double Protein Expression Lymphoma; TPL: Triple Protein Expression Lymphoma.

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Authors' Contributions

Project leader and study design: Chang KM. Wrote the paper and bioinformatics analysis: Ting CY. Co-wrote the manuscript: Kuan JW. Performed immunohistochemistry testing and FISH testing: Ting CY. Slides review and performed Histopathology analysis: Samsudin AT, Wong JOL, Yusuf Y, Raja Gopal N, Puri R, Bahari SK. Data collection: Wong LLL, Chew LP, Lee SK, Ong TC, Goh AS and Teoh CS, Mohd Nurjaya.

Availability of data and materials

All test methods and data analysed in this study are presented in this article. Results and laboratory findings are tabulated in the supplementary file 1, supplementary file 2 and supplementary file 3.

Competing Interests

The authors have declared that no competing interest exists.

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