ORIGINAL ARTICLE

# Classifying DLBCL according cell of origin using Hans algorithm and its association with clinicopathological parameters: A single centre experience

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**ABSTRACT**

**Introduction: In recent years, "double hit" and "double protein" involving gene rearrangement and protein expression of c-MYC and BCL2 and/or BCL6 are the most used terms to describe poor prognostic factors in diffuse large B-cell lymphoma (DLBCL). This study was to determine the frequency of double or triple protein expression by using immunohistochemistry (IHC) and comparing the result with clinicopathological features and cell of origin (COO) classification.**

**Methods: We conducted a cross-sectional study by using 29 archived formalin-fixed paraffin embedded tissue blocks of DLBCL. All the samples were evaluated for the subgrouping of COO DLBCL was determined by expression of CD10, BCL6 and MUM1 based on Hans classification. In addition, expressions of c-MYC, BCL2 and BCL6 were detected by IHC.**

**Results: Among the 29 cases, MYC, BCL2 and BCL6 proteins were detected in 72.4%, 62.1% and 62.1% of patients, respectively. Concurrent expression (c-MYC positive/BCL2 positive and/or BCL6 positive) was present in 58.6% of patients. 34.5% were categorised as germinal centre like (GCB) subgroup and 65.5% were categorised as non-germinal centre like (non-GCB) subgroup. Among the clinicopathological features, the double/triple protein expression lymphoma was significantly associated with elevated LDH level (p=0.018), IPI score (p=0.003), Ann Arbor stage (p=0.011) and complete response rate (p=0.011).**

**Conclusion: Double/triple protein lymphoma was strongly associated more adverse clinical risk factors. Thus, analyses of MYC, BCL2 and BCL6 expression by IHC represents a rapid and inexpensive approach to risk-stratify patients with DLBCL at diagnosis.**

**KEY WORDS:**
*DLBCL, HANS ALGORITHM, GC, NON-GC*

## INTRODUCTION

National Cancer Registry 2016 of Malaysia reported that lymphoma is the fourth most common cancer regardless of gender. It is the fourth (5.5%) and sixth (3.9%) most common cancer in males and females respectively. Malay males had the higher rate of lymphoma incidence as compared to the Chinese and Indians. In Kelantan, it was reported that the age standardised incidence of lymphoma among males and females is estimated at 3.1 in males and 2.4 in females each per 100,000 people.[1] Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphomas (NHL) comprising up to 40% of cases globally.[2] DLBCL was reported as 60% of all NHL in Malaysia as published in the reports in 1999 and 2003.[3,4] A report from one centre in Malaysia, stated that 46% of the NHL cases that they received were DLBCL.[5]

DLBCL is a biologically and clinically heterogeneous group of B-cell lymphomas with wide variation in patient survival. The prognosis of DLBCL depends on the clinical features such as International Prognostic Index (IPI)[6] and gene expression profiling. Many studies have been reported of the variable prognostic measures of DLBCL.[7,8] DLBCL have been categorised into prognostically significant subgroups, using cDNA microarray into germinal centre B cells (GCB) and activated B cells (ABC).[9] It is impractical and expensive to perform microarray analysis on every patient with DLBCL, thus various immunohistochemical (IHC) algorithms have been developed to translate the robust information from molecular studies into a routine clinical platform.[10-14]

Algorithms developed by Hans et al., using immunohistochemistry was first widely accepted as a mechanism to divide DLBCL into germinal centre (GC) and non-GC subtypes.[11] The algorithm is based on IHC expressions of CD10 (Cluster of Differentiation 10), BCL6 (B Cell Lymphoma 6) and MUM1(Multiple Myeloma 1) proteins. The advantage of using Hans IHC algorithm is that it uses only three easily assessable antibodies, which made it widely acceptable as compared to other algorithms that were developed later to subtype DLBCL according to the cell of origin (COO). Studies showed that Hans algorithm correlates well with the corresponding gene expression profile results[10,11] and showed clear survival differences between the GCB and ABC DLBCL groups.[11] Since then, many studies have been published, some of were concordance with the prognostic division according to Hans,[4,15] whilst others did not find statistically significant differences between these two groups.[16,17]

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**Table I: Clinicopathological features of cell of origin (n=29)**

| VARIABLE | TOTAL NO. | COO | | p-value |
|---|---|---|---|---|
| | | GCB n = 10 n (%) | NON-GCB n=19 n (%) | |
| AGE | | | | 0.245[a] |
| >60 YEARS OLD | 14 | 3 (21.4) | 11(78.6) | |
| ≤60 YEARS OLD | 15 | 7 (46.7) | 8 (53.3) | |
| GENDER | | | | 0.27[a] |
| MALE | 13 | 6 (46.2) | 7 (53.8) | |
| FEMALE | 16 | 4 (25) | 12 (75) | |
| ETHNIC | | | | 0.532[a] |
| MALAY | 27 | 10 (37) | 17 (63) | |
| CHINESE | 2 | 0 (0) | 2 (100) | |
| PERFORMANCE STATUS | | | | 0.665[a] |
| 0 TO 1 | 22 | 7 (31.8) | 15(68.2) | |
| 2 TO 5 | 7 | 3 (42.9) | 4 (57.1) | |
| ELEVATED LDH | 18 | 6 (33.3) | 12(66.7) | 1.000[a] |
| YES | | | | |
| EXTRANODAL SITE ≥2 | 9 | 3 (33.3) | 6 (66.7) | 1.000[a] |
| ANN ARBOR STAGING | | | | 0.665[a] |
| I/II | 7 | 3 (42.9) | 4 (57.1) | |
| III/IV | 22 | 7 (31.8) | 15(68.2) | |
| IPI SCORE | | | | 0.126[a] |
| 0 TO 2 | 17 | 8 (47.1) | 9 (52.9) | |
| 3 TO 5 | 12 | 2 (16.7) | 10(83.3) | |
| COMPLETE RESPONSE RATE | | | 0.665a | |
| YES | 7 | 3 (42.9) | 4 (57.1) | |
| NO | 22 | 7 (31.8) | 15(68.2) | |

a Fisher-exact test p-value

This cell of origin subtyping concept does not identify individual patients who will suffer an aggressive clinical course, because these patients can be found in both the subgroups. This study is to classify DLBCL into GC or non-GC according to Hans algorithm, and their association with clinicopathological parameters in patients presented to Hospital Universiti Sains Malaysia (HUSM).

## MATERIALS AND METHODS

*Patients selection*

There were 192 cases of DLBCL reported from the beginning of 2004 to early 2015, excluding trephine cases. Cases with unavailable sufficient clinical data, missing or insufficient tissue blocks, transformed cases from low grade, overlapping cases and external cases were excluded from. In all 29 cases of de novo DLBCL fulfilled the selection criteria. All cases were diagnosed according to World Health Organization (WHO) 2008 classification criteria.[18] Formalin fixed paraffin-embedded tissue blocks archived in the Department of Pathology, HUSM were retrieved. The cases included nodal and extra-nodal DLBCL. The clinicopathological data consist of age, performance status, extra-nodal involvement, serum LDH (lactate dehydrogenase) level, Ann Arbor stage and IPI were retrieved from the original archived formal pathology reports and patients' case notes.

*Immunohistochemical investigation*

All tissue biopsies were fixed routinely in 10% buffered formalin, embedded in paraffin, and cut into 3µm sections. Following deparaffinisation, heat-induced antigen retrieval techniques were used. For this, the sections were placed in a pressure cooker with Tris buffer (1mmol/L EDTA (Ethylene Diaminetetra Acetic Acid), pH9.0) for 3 minutes after reaching operating temperature and pressure. Endogenous peroxidase activity was then blocked with 3% H2O2. Primary antibodies; CD10 (DAKO USA) and MUM1 (DAKO USA), both at dilution of 1:50 were applied to the sections and incubated for 1 hour at room temperature. Detection of the primary antibody was performed by using Horseradish peroxidase (HRP) polymer solution (DAKO Real Envision Detection System). Tonsils with reactive lymphoid hyperplasia served as an external control tissue for CD10 and MUM1. For BCL6, after using the similar heat-induced antigen retrieval technique, the section was incubated with the primary antibody for 30 minutes at room temperature. Followed by endogenous peroxidase blocking with 3% $H_2O_2$. The section was again incubated with DAKO Mouse Linker for 20 minutes before application of secondary antibody.

*Immunohistochemical scoring*

The IHC stained sections were evaluated by WN and FAH independently. Disagreements were resolved by joint review on a multi-headed microscope.

COO was assessed according to the Hans criteria.[11] In Hans criteria cases were considered positive if 30% or more of the tumour cells were stained with the antibody based on previously established cut-off point. Hans algorithm was made up of three markers (CD10: GC marker; BCL6: associated with both GC and non-GC subtype; MUM1: post GC marker). CD10 (clone 56C6) expression showed cell surface membrane pattern, BLC6 (clone PG-B6P) and MUM1 (clone MUM1p) expressions exhibit distinct nuclear pattern. Based on combination of the three markers, Hans algorithm could divide DLBCL into two subtypes: GC and non-GC. The

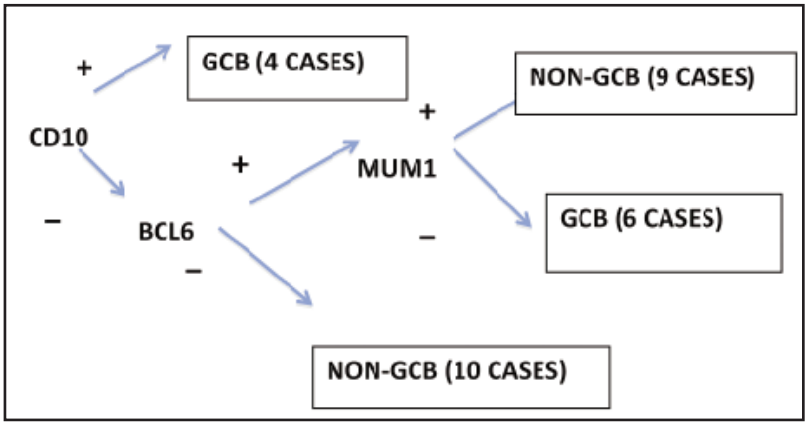


**Fig. 1:** Results using classification of cell of origin (COO) based on Hans algorithm.

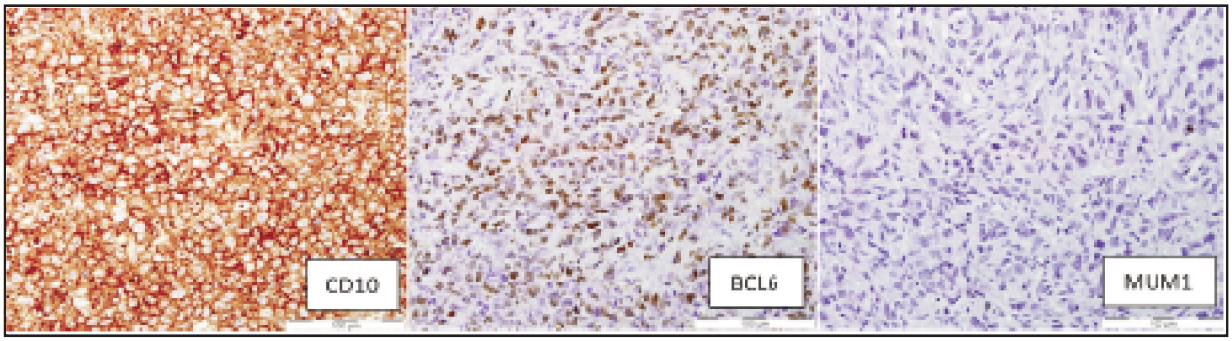


**Fig. 2:** Results of IHC staining, Germinal Centre type DLBCL. Positive for CD10 and BCL6 but negative for MUM1. Original magnification, x400.

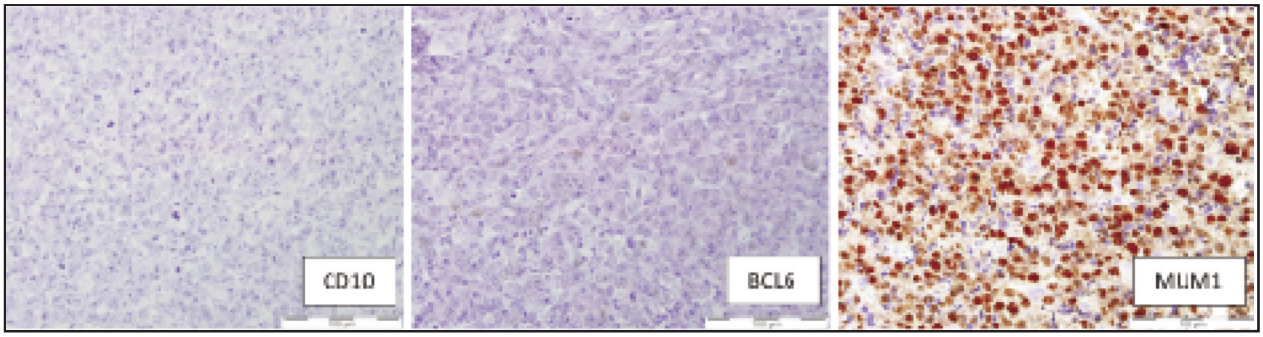


**Fig. 3:** Results of IHC staining, Non-Germinal Centre type DLBCL. Negative for CD10 and BCL6 but positive for MUM1. Original magnification, x400.

COO GC were those cases that were immunoreactive to CD10, BCL6 and negative for MUM1. Whilst COO non-GC cases were immunoreactive to MUM1, can be either positive or negative to BCL6, and absolutely negative for CD10.

All DLBCL patients in our centre were treated with the R-CHOP regimen (Rituximab, Cyclophosphamide, Adriablastine, Vincristine, Prednisone) at standard dosage as prescribed by AH.

Statistical analysis was conducted using SPSS22.0 software. Characteristics of COO and clinicopathological parameters were analysed using the chi-square test. Association were considered statistically significant when p value of ≤0.05.

## RESULTS

A total of 29 cases of de-novo DLBCL were selected from the 192 cases, that fulfilled the inclusion criteria which were availability and adequate tissue blocks, non-overlapped cases and complete clinical data. The patients included 13 males and 16 females with a median age of 57.3 years (range, 1-78 years). Majority of the patients were Malays (93.1%) and then followed by Chinese (6.9%). The samples were from lymph node (15 cases) and extra-nodal tissue (one each from large intestine, mandible, mediastinum, ovary, soft tissue, thyroid and uterus; two each from oral mucosa and testis; and, three from tonsil)

*Cell of origin*

Expression of CD10 was observed in 13.8% (4 of 29) of the patients, BCL6 in 62% (18 of 29) and MUM1 in 51.7% (15 of 29). Of the 29 cases, 10 (34.5%) were considered GC and 19 (65.5%) were considered non-GC by immunohistochemical analysis (Figure 1). The cases were assigned to the GC group if CD10 alone was positive or, if both BCL6 and CD10 were positive (Figure 2). Of the GC cases, three cases showed expressions of both CD10 and BCL6, 1 case expressed CD10 alone and 6 cases expressed BCL6 alone (CD10-, MUM1-). The cases were assigned to the non-GC group if positive for BCL6 and MUM1 and CD10 negative or only MUM1 was positive (Figure 3).

The clinicopathological features of the patient with GC and non-GC are summarised in Table I. The two subtypes of DLBCL, GC and non-GC did not differ with regard to any of the clinical features ($p>0.05$).

## DISCUSSION

DLBCL is the most common type of NHL and considered an aggressive lymphoma. Predicting prognosis in an individual patient is very difficult as DLBCL comprises a group of morphologically, immunohistochemically, and clinically heterogeneous tumours rather than one single entity.[19] The application of gene expression technology is impractical for routine clinical use because of the cost, requiring fresh tissue and specialised skills. Therefore, with the changes in antigen retrieval techniques, commercialization of antibodies, and automation of staining, immunohistochemistry is presently the cheaper option to determine cell of origin of DLBCL.

The population of Kelantan comprised of Malay 94.6%, Chinese 3.3%, Indian 0.3%, others (including other Bumiputera) 1.8%.[1] Thus, the study is likely to be bias of one set of ethnic background only, which may not represent the whole population of Malaysia which is of more diverse ethnicities. The median age in this study was 57.3 years, as compared to other Malaysian DLBCL studies, the median age of 54.1[20] and 63.[21] Rather this is still considered as among the younger age compared to the western DLBCL population.[22] The age of 1-year-old patient included in the study is rather controversial as DLBCL is rare in infant. Re-evaluation of the case should be considered. Our study also showed that fewer males (13) compared to females (16) are affected by DLBCL which differ from finding of other local DLBCL by Ting et al., that showed male: female ratio of 1.14:1.[20]

DLBCL is also known to present about 40%[23] as primary extra-nodal lesions, which was reported in this study (48.2%). Similar findings were also demonstrated by Ting et al., that showed 42.5%[20] and Masir et al., 41%.[22] The prevalence of GC 10 (34.5%) and non-GC 19 (65.5%) in our cohort is comparable to other local DLBCL studies, (GC40%; non-GC 60%)[20] and (GC 40%; non-GC 60%).[21] The higher incidence of extra-nodal lymphoma in our cohort may have influenced the reversed non-GC distribution as compared to the West.[18]
We were unable to demonstrate significant association between clinicopathological factors and cell of origin: GC and non-GC. These findings are similar to Hans et al., wherein those two groups of DLBCL: GC and non-GC did not differ to any of the clinical features.[11] This showed that the cell of origin sub-classification unable to identify individual patients who will have the adverse clinical risk factors.

Other IHC algorithms such as Choi, Tally, Nyman, Nakunam[14] and Muris[13] with attempts to improve the predictive significance using antibodies. However, Hans is still more often used because of only three antibodies are required to sub-classify DLBCL according to their COO. In a study by Meyer et al.,[14] regarding the IHC methods for predicting cell of origin and survival in patients with DLBCL treated with Rituximab, they found the Choi[10] and Hans algorithms[11] had high concordance with the microarray results. This study tried to remove BCL6 and formed modified Hans* and Choi* algorithms, which showed retained high concordance with the microarray results as the original algorithms. Some centres in Malaysia only utilise two antibodies which are CD10 and MUM1. BCL6 is often been dropped because BCL6 technically is difficult to perform and as it results in difficulty in interpretation.[23] In our study, we used monoclonal antibody clone PG-B6p to detect BCL6 molecule in tissue sample. Along with this antibody, we used Dako Mouse Linker which successfully eliminated the background staining. Therefore, we retained the BCL6 as in the Hans algorithm.

In a study by Benesova et al., they argue that Hans algorithm has failed to discriminate GC and non-GC in terms of different survival probability with immunochemotherapy treatment.[17] Similar conclusions were also made in another study.[24] Our study also, showed that there is no statistical significance of complete response rate between the two subtypes. This is similar to findings of local DLBCL study.[20] However, compared to other published studies, our study has a small number of samples which may affect the statistical analysis. However, some studies showed that Hans algorithm is useful and can separate DLBCL patients into prognostic groups.[14] The Hans algorithm is not a perfect substitute for gene expression profile in predicting the disease prognosis, but it is a substitute for identifying COO in centres with limited resources, which then provide invaluable information to the treating clinicians. Hence this will comply with the current WHO mandatory COO classification of DLBCL for patient. Other factors that need to be taken into account is that different namely, IHC techniques, may result in variable results and poor reproducibility for almost all markers. Furthermore, inter- and intra-observer variations may inevitably influence the results.

This study also did not discuss the survival of the patients which needs to be explored further. By including other centres from east coast peninsular Malaysia, a higher number of samples can be recruited and may represent a more diverse and statistically significant results. Nevertheless, the study had provided an important information from one single centre at HUSM.

## ETHICAL APPROVAL

Approval for the study was obtained from the Human Research Ethics Committee USM (HREC) with the code number: USM/JEPeM/1609280

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## CONFLICT OF INTEREST

We declare that there is no conflict of interests regarding the publication of this article.

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