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Original Article

Pathology Section

Immunohistochemical Characterisation of Cell of Origin in Diffuse Large B-cell Lymphoma and its Association with the Double Expressor Phenotype: A Retrospective Study

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ABSTRACT

Introduction: Diffuse Large B-cell Lymphoma (DLBCL) is the most common type of Non-Hodgkin Lymphoma (NHL) and is categorised into the Germinal Centre B-cell (GCB) and Activated B-cell (ABC/Non GCB) subtypes as per the Cell Of Origin (COO) model with the help of gene expression profiling/immunohistochemistry. The non GCB subtype has been found to associate with the Double Expressor (DE) phenotype (i.e., co-expression of BCL-2 and c-myc by IHC and this association was substantiated and proven to be statistically significant in the present study.

Aim: To categorise all DLBCL cases into GCB and Non GCB and further into DE and Non-DE by using Hans and Choi IHC alogrithm.

Materials and Methods: A retrospective study of 50 patients was carried out with the help of archival material filed in the Department of Anatomic Pathology at Dharamshila Narayana Superspeciality Hospital, New Delhi, India from 1st January 2019 to 30th June 2020. The study was approved by Instituitional Ethics Committee (IEC). The study cohort was divided into two groups- group A with nodal presentation and group B with extranodal presentation. By using the Hans and Choi IHC algorithms, the cases were categorised into the GCB and non GCB subtypes in both the groups. Chi-square test and Yates correction were used for statistical analysis.

Results: The median age at presentation was 49 years (20-81 years) with a male to female ratio of 2.3:1 (35 males and 15 females). Group A with nodal disease included 28 patients and group B with extranodal disease included 22 patients. The DE phenotype was determined in each case by the co-expression of c-myc (>40%) and BCL-2 (>50%) by IHC. By using the statistical chi-square test analysis and Yates correction, the association of DE was found to be statistically significant with non GCB type lymphomas and non DE with GCB lymphomas with p-value being 0.0005 and 0.00168, respectively.

Conclusion: Due to the heterogeneity inherent in DLBCL, prediction of the DE phenotype and the COO by IHC is a sensitive tool and helps in the prognostication and therapeutic triage of patients. Hence, in all the cases diagnosed as DLBCL, a detailed morphological and IHC work up is mandatory to determine prognosis and possibly tailor therapy according to COO and DE phenotype.

Keywords: Cell of origin model, Hans and choi algorithm, Immunohistochemistry

INTRODUCTION

The DLBCL is the most common type of aggressive NHL, representing approximately 24% of all newly diagnosed cases of NHL. It represents a heterogenous group of diseases having variable outcomes that are differentially characterised by clinical features and most recently by recurring mutations [1,2]. Clinically, patients present with rapidly enlarging lymphadenopathy, along with constitutional symptoms. There is a high frequency of extranodal disease, if there is generalised lymphadenopathy [1,2].

The "COO" model divides DLBCL into the GCB type and non GCB type or ABC type depending upon gene expression pattern. The gene expression of the germinal centre type fits with the normal germinal centre derived B cell and in the subtype non GCB DLBCL or ABC lymphoma, the gene expression profiling more closely fits in with a normal ABC [1,2]. The COO model also determines the prognosis of the two biological subtypes of DLBCL that should be treated differently. C-myc is a proto-oncogene on chromosome 8q24 and encodes a transcription factor, which leads to cellular survival and proliferation, if dysregulated. The BCL-2 is an oncogene with an antiapoptotic property [1,2].

Myc rearrangement t(8;14) is prototypically associated with Burkitt lymphoma but is also associated with 12-15% of DLBCL. The BCL-2 rearrangement t(14;18) is also important as it leads to a drug resistant phenotype with increase in cancer cell survival. Thus a concurrent rearrangement of BCL-2 and c-myc, which are present in approximately 5-7% of DLBCL, leads to a clinically resistant form of DLBCL, termed as a double hit lymphoma and is associated with a poor prognosis [3]. The revised WHO classification recognises the co-expression of myc (>40%) and BCL-2 (>50%) proteins as a new adverse prognostic marker within DLBCL, Not Otherwise Specified (NOS) as "DE lymphoma" [4-6].

Another variant of the double hit lymphoma presents with co-rearrangement of c-myc and BCL-6 genes. All the three genes BCL-2, myc and BCL-6 are simultaneously rearranged in the phenotype termed as "Triple-Hit Lymphoma (THL)" [6]. Patient who have the double hit rearrangement usually have protein over-expression and therefore have the DE phenotype. However, the converse is not always true [7]. Double hit lymphoma occurs more commonly in GCB type of DLBCL, while DE lymphoma is commoner in the non GCB type of DLBCL. DEs are detected by IHC staining and double hit lymphomas are detected by Fluorescence In Situ Hybridization (FISH) [7].

The most common upfront treatment for DE lymphomas is Chemoimmunotherapy (CI) with R-CHOP (Rituximab, Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone) which leads to a cure in 50-60% of patients. Patients, who develop disease that is refractory to upfront treatment or relapse after achieving remission, are treated with Autologous Stem Cell Transplantation (ASCT) and they generally have a poor outcome.

The majority of patients with DE lymphoma relapse after R-CHOP [8], hence such patients should be treated with ASCT. For patients unable to undergo transplantation, the median survival is six months. Chimeric Antigen Receptor T-cell Therapy (CART) is promising for patients with aggressive BCL that do not respond to other treatment modalities.

The main aim and objective of present study was that all high grade DLBL should be categorised into their molecular subtypes i.e., GCB lymphomas and non GCB lymphomas and further into DE and non DE phenotypes by using Hans and Choi Alogrithm (IHC Alogrithm) [9,10], as it aids in risk stratification, prognostication and for appropriate treatment, as it varies for each subtype. Therefore, all patients of DLBCL should be assessed for the DE phenotype, which aids in risk stratification of the patient and further helps in optimising the treatment strategy.

MATERIALS AND METHODS

This was a retrospective study conducted at Dharamshila Narayana Superspeciality Hospital, New Delhi, India from 1st January 2019 to 30th June 2020, to validate a model to predict the DE phenotype based on COO subtype. The study was approved by Institutional Ethics Committee (IEC), (name of committee-Dharamshila Narayana Superspeciality Hospital) vide registration no. ECR/226/Inst/DL/2019. Written consent was obtained.

Inclusion criteria: The study includes all high grade DLBCL cases.

Exclusion criteria: Excludes other high grade non-hodgkin lymphomas.

Study Procedure

All newly diagnosed DLBCL patients were divided into group A including patients with nodal involvement and group B including patients with extranodal involvement. Based on "COO" both the groups were further divided into GCB type and non GCB type of DLBCL. With the help of IHC using the Hans or Choi algorithm IHC staining for BCL-2 and c-myc was used to determine the DE phenotype and its association with the COO subtypes of DLBCL. Present study included 50 patients, who presented with generalised lymphadenopathy and/or extranodal disease along with other associated constitutional symptoms. A biopsy was performed in each case and sent in 10% neutral buffered formalin to the Department of Surgical Pathology for further processing and evaluation. Formalin fixed paraffin embedded tissue sections were cut and mounted on slides and stained with routine Haematoxylin and Eosin (H&E) stain.

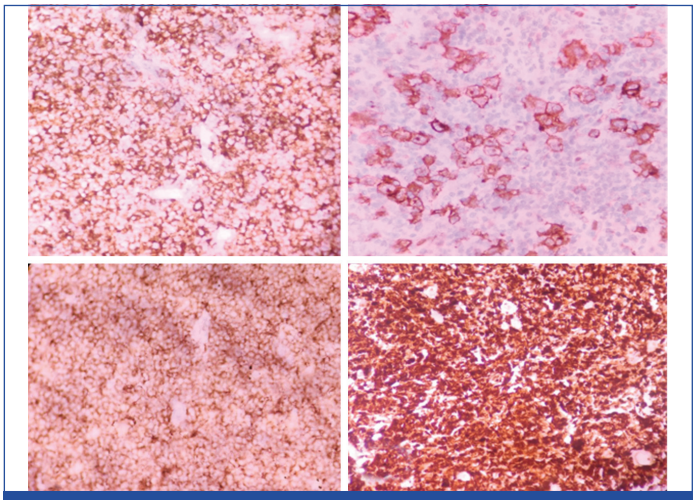
Further, IHC stains were applied to assess the 'COO' and DE phenotypes based on the percentage of staining of the cells and were analysed. In all cases IHC was performed using the automated Roche Ventana Benchmark XT IHC Autostainer (Ventana Medical Systems Inc. Tucson, Arizona) with the aid of the UltraView Universal DAB Detection Kit. The antibodies and clones employed were- CD3 (PS-1), CD5 (4C-7), CD10 (56C6), CD20 (L-26), CD30 (BerH2), BCL-2 (EP-36), BCL-6 (EP278), C-myc (EP121), MUM-1 (EP190), Ki-67 (30-9) and FOXP-1 (EP137) provided by Pathnsitu Biotechnologies Pvt. Ltd. and GCET-1(ab68880 from ABCAM (Cambridge, UK). Coexpression of c-myc (>/=40%) and BCL-2 (>50%) in neoplastic cells by IHC staining was considered as DE phenotype.

STATISTICAL ANALYSIS

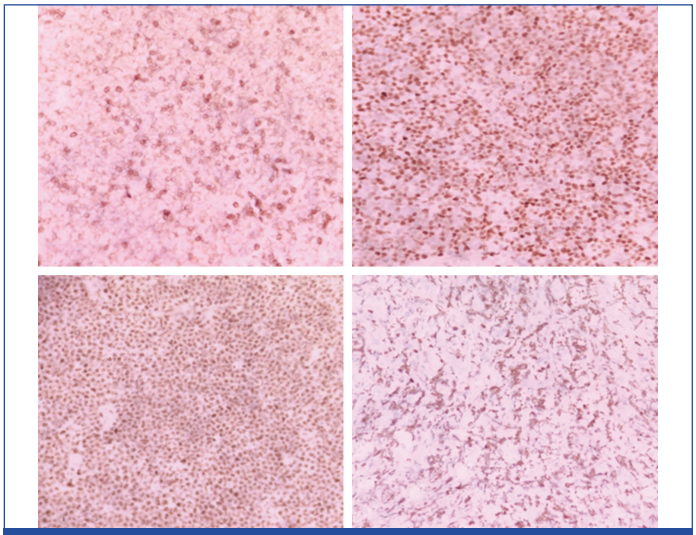
The statistical analysis used in present study was Chi-square test and Yates Correction to calculate the p-value and also to determine the significance of different parameters.

RESULTS

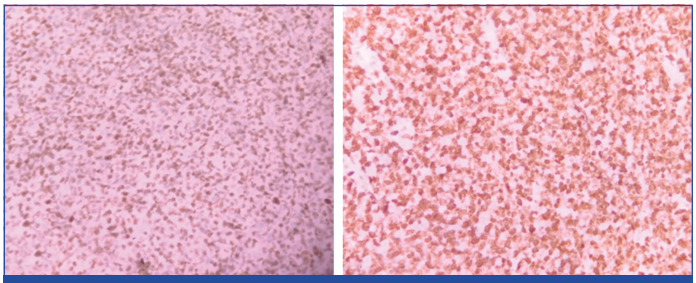
The median age at presentation was 49 years (20-81 years) with a male to female ratio of 2.3:1 (35 males and 15 females). Group A with nodal disease included 28 patients and group B with extranodal disease included 22 patients, constituting 56% and 44%, respectively of the study cohort. Histological sections examined from biopsy specimens showed diffuse infiltration by predominantly large atypical lymphoid cells with prominent nucleoli and mitotic activity. All cases were positive for the pan B-cell marker CD20 and a few showed positivity for CD30. Eight of the 28 patients in group A were categorised as GCB type and 20 (71.4%) were categorised as non GCB type by immunohistochemistry, using the Hans and Choi algorithm. Out of the eight GCB type, only 1 (12.5%) had a DE phenotype, while 7 (87.5%) were non DE type. Out of the 20 non GCB type, 15 (75%) had a DE phenotype and 5 (25%) were of the non DE type. The features of immunostaining can be appreciated in [Table/Fig-1-10].



[Table/Fig-1]: Diffuse strong membrane staining for CD20 in lymphoma cells (x400). **[Table/Fig-2]:** Patchy membrane and golgi zone staining for CD30 seen in some cases (x400). **[Table/Fig-3]:** Diffuse membrane staining for CD10 in tumour cells in DLBCL, GCB type (x400). **[Table/Fig-4]:** Diffuse strong granular cytoplasmic staining for GCET-1 in DLBCL, GCB type (x400). (Images from left to right)



[Table/Fig-5]: Diffuse staining for BCL-2 in > 50% lymphoma cells in Double Expressor (DE) type DLBCL (X400). **[Table/Fig-6]:** Diffuse nuclear staining for c-myc in >40% lymphoma cells in Double Expressor (DE) type DLBCL (X400). **[Table/Fig-7]:** Diffuse nuclear staining for FOXP-1 in tumour cells in Non GCB type DLBCL (X400). **[Table/Fig-8]:** Nuclear staining for MUM-1 in tumour cells in non GCB type DLBCL(X400). (Images from left to right)



[Table/Fig-9]: A. Diffuse strong nuclear staining for BCL-6 in tumour cells in GCB type DLBCL (X400). **[Table/Fig-10]:** High Ki-67 labelling index (80-90%) in lymphoma cells (x400). (Images from left to right)

Four of the 22 patients in group B (18.2%) were categorised as GCB type and 18 (81.8%) as non GCB type. Out of the four GCB type,

none showed a DE phenotype. However, 10 of the 18 non GCB type (55.6%) were DE type and 8 (44.4%) were non DE type. The DE phenotype was present in 26/50 (52%) and the NDE phenotype was expressed in 24/50 (48%) of cases included in the present study. While in the non GCB type DE phenotype was expressed in 25 cases (65.8%) out of 38 cases, only one of the 12 GCB cases expressed the DE phenotype (8.3) [Table/Fig-11-13].

Nodal Group A (28 patients)			Chi-square value=9.115 Df=1 p-value=0.002 Yates correction=6.741 p-value=0.009 Significant
GCB (8 cases)		Non GCB (ABC) (20 cases)	
DE phenotype	1 (12.5%)	15 (75%)*	
NDE phenotype	7 (87.5%)	5 (25%)	

[Table/Fig-11]: Incidence of DE and NDE phenotype in the GCB and non GCB subtype in Group A.
*value in parenthesis shows percentage

Extra-Nodal Group B (22 patients)			Chi-square value=4.074 Df=1 p-value=0.04 Yates correction=2.141 p-value=0.1434 Non significant
GCB (4 cases)		Non GCB (18 cases)	
DE phenotype	0	10 (55.6%)*	
NDE phenotype	4 (100%)	8 (44.4%)	

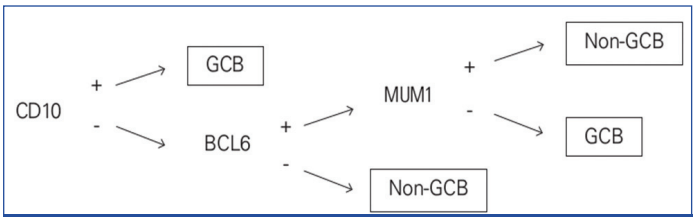
[Table/Fig-12]: Incidence of DE and NDE phenotype in the GCB and non GCB subtypes in Group B.
*value in parenthesis shows percentage

Total patients (50)			Chi-square value=12.062 Df=1 p-value=0.0005 Yates correction=9.87 p-value=0.00168 Significant
GCB (12 cases)		Non GCB (38 cases)	
DE phenotype	1 (8.3%)	25 (65.8%)*	
NDE phenotype	11 (91.7%)	13 (34.2%)	

[Table/Fig-13]: Overall Incidence of DE and NDE phenotype in the GCB and non GCB subtype.
*value in parenthesis shows percentage

Thus, in the nodal group the non GCB subtype analysed with the DE phenotype and the association was found to be statistically significant (p-0.009). In the extranodal group, though the non GCB subtype showed the DE phenotype more commonly than the NDE phenotype, the association was not found to be statistically significant (p-0.1434). Overall the DE phenotype analysed with the non GCB subtype and the NDE phenotype with the GCB subtype. The association was found to be statistically significant.

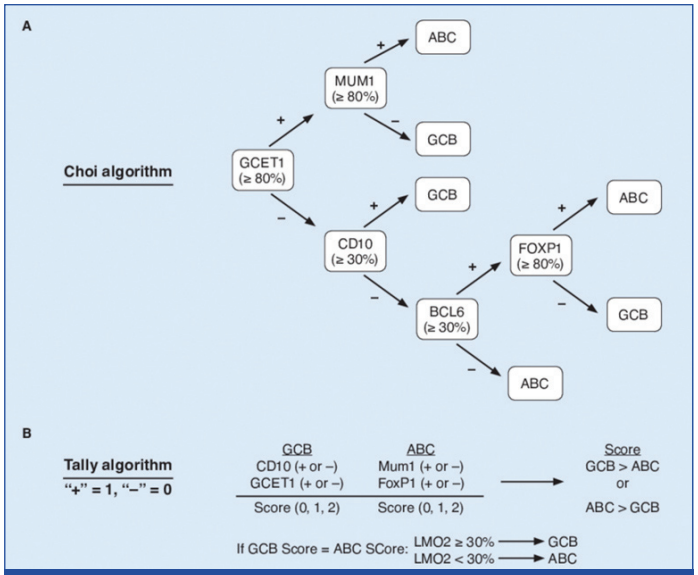
Thus, the DE phenotype associated with the non GCB subtype and the NDE phenotype with the GCB subtype in this study. The study has used the IHC algorithm (Hans and Choi) to do the molecular subtyping of all lymphoma cases. The co-expression of c-myc (>40%) and BCL-2 (>50%) proteins in DLBCL, NOS cases or any of the above one with BCL-6 (>30%) is considered as "DE lymphoma" [Table/Fig-14,15] [9,10].



[Table/Fig-14]: Hans Algorithm to approximate molecular subtypes based on immunohistochemical patterns. GCB- Germinal Centre B-cell Like [9].

DISCUSSION

According to the 2016, World Health Organisation (WHO) classification, diagnosis of all cases of DLBCL, NOS should include COO, (GCB vs. ABC or non GCB, if an IHC algorithm is used), because of their different molecular features, biologic behaviour, prognosis and treatment [3]. This distinction is important because better outcomes are seen in patients with the germinal-center subtype than with the non germinal-center subtype. We therefore now have a "COO" model showing that there are at least two genetic biologic types of DLBCL i.e., GCB



[Table/Fig-15]: Choi and Tally Algorithm to determine cell-of-origin based on Immunohistochemistry [10].

and non GCB subtype that explains the prognosis and is targeted differently. This insight has led to recent trials that test new treatments in one genetic subtype vs the other. We are hopefully very close to understanding whether these two subtypes of DLBCL by using two different IHC algorithm (Hans CP et al., and Choi WWL et al.,) [9,10] and should be treated differently. The most challenging profile to detect in routine practice is COO. The gold standard for identifying germinal-centre DLBCL versus non germinal-centre DLBCL was first defined as gene-expression profiling patterns in frozen tumour material. However, gene-expression profiling is not routinely available nor is it considered a standard test. The most common approach is IHC using different algorithms, such as the Hans, Choi or Tally algorithms, to determine whether a lymphoma is germinal-centre DLBCL or non germinal-centre DLBCL. As compared to gene expression profiling in frozen material, there is an error rate of approximately 20% with immunohistochemical algorithms shown below [11]. The present study has used the Hans/ Choi Algorithms to determine the COO.

The presence of both the myc and BCL-2 rearrangements defines Double-Hit Lymphoma (DHL). This phenotype is very proliferative and drug-resistant, and it is associated with a poor prognosis. Another variant of DHL is co-rearrangement of myc and the BCL-6 gene. Rarely, all three genes BCL-2, Bcl-6 and C-myc, patients who have the double-hit rearrangement usually have protein overexpression, and therefore have the DE phenotype. However, the converse is not always true: dual-expressor protein overexpression is not always associated with an underlying double-hit re-arrangement. Complicating the picture is that most DHLs occur in the setting of a germinal-centre DLBCL, whereas most DE lymphomas occur in non-germinal-centre DLBCL as demonstrated in this study which shows a strong association between the DE phenotype and the Non GCB COO subtype by IHC [12,13]. myc and BCL-6 are simultaneously rearranged in a phenotype termed THL. Both double-hit and THL s have a poor prognosis with standard treatment [14]. The starting point in a discussion about prognostic variables in DLBL is the observation that some patients can be cured and others cannot. Several studies have investigated the biologic underpinnings for these different outcomes. A pioneering genetic evaluation of DLBCL, published by Alizadeh AA et al., showed two subtypes: germinal center and non germinal center (also known as ABC) [15].

The IHC staining to identify protein expression of myc also showed that there are lymphomas in which myc and BCL-2 genes are overexpressed at a protein level, without the genetic rearrangements. This profile has been referred to as the "DE" phenotype in DLBCL in the revised 2016 WHO classification of lymphoid neoplasms. The WHO classification defines overexpression as greater than 40%

c-myc-expressing cells and greater than 50% BCL-2-expressing cells by IHC [3]. As shown in a study by Hu S et al., patients with DE DLBCL have worse outcomes than patients in whom these proteins are not overexpressed; in general, only one-third of patients have long-term disease control with R-CHOP [16].

DHL is relatively uncommon, occurring in approximately 5-7% of patients with DLBCL. However, DE lymphomas may be present in as many as one-third of patients with DLBCL and serve to identify a significant subset of cases with a worse prognosis. The DHL's can be detected with FISH or standard cytogenetic analysis. The DE lymphomas are diagnosed by IHC. The DE phenotype was not given a unique category, but was recognised by the WHO as a poor prognostic sign within DLBCL [3].

When patients with aggressive B-cell lymphomas relapse or become refractory to therapy, standard options are limited. For patients unable to undergo transplant, or for those who relapse after a stem cell transplant, the median survival is approximately six months. Despite the many trials that have tried to improve upon this dismal statistic, there are no breakthroughs at this time. Chimeric Antigen Receptor T-cell therapy (CART) is exciting. This treatment is still in the early phases of research and associated with toxicity, but it is promising for patients with aggressive B-cell lymphomas that do not respond to other therapies. There are also a number of new biologic and targeted agents that are promising, and finding which patients may respond to a particular treatment is a high priority [17].

Limitation(s)

The limitation of present study was that, there was an approximately 20% error rate with IHC algorithm. The most sensitive is always gene expression profiling/m-RNA based technique, which is not usually available in a limited setting facility. Hence, Immunohistochemistry is routinely done to categorise all the high grade B-cell lymphomas into their molecular subtypes as an alternative solution.

CONCLUSION(S)

The distinction of GCB versus ABC-DLBCL has led to differences in primary treatment by emerging new-targeted therapy. The current standard of care for most patients is R-CHOP, which has improved dramatically the outcome of DLBCL. However, for patients who fails to respond to R-CHOP, the choice of therapy is very likely to be influenced by the COO and the molecular pathways used by the tumours for survival and proliferation.

Although there are no strict recommendations on how to select cases for FISH analysis, a reasonable approach is to perform FISH analysis for myc, BCL-2 and/or BCL-6 in cases with aggressive clinical presentation, GCB phenotype, and double expression of myc and BCL-2 which has been shown to associate with the non GCB subtype as demonstrated by this study, thereby mandating a comprehensive and detailed morphologic and IHC work-up in all cases of high GBL in order to determine prognostic and therapeutically relevant and biologically distinct phenotypes.

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