ORIGINAL ARTICLE

Double Hit and Double Expresser Diffuse Large B Cell Lymphoma Subtypes: Discrete Subtypes and Major Predictors of Overall Survival

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Abstract Double hit lymphomas (DHL) and double expresser lymphomas (DEL) are subsets of diffuse large B cell lymphomas (DLBCL) which are being increasingly recognised as cause of treatment failure. This emphasizes the need for their separation from other DLBCL cases in order to prognosticate and administer more aggressive treatment to this set of patients. The present study was conducted with the aim to identify the DHL/DEL patients and study their distinctive clinicopathological profile and overall survival. This retrospective analysis involved 172 cases of DLBCL sub-classified on the basis of cell of origin. Immunohistochemical (IHC) analysis for *MYC, BCL2, BCL6, MUM1* and CD10 was performed. Rearrangement studies were performed using break apart Fluorescent in situ hybridization. Overall survival (OS) was also evaluated. Distinctive clinical and pathological features of DHL and DEL were identified. Rearrangement study by FISH revealed seven cases of DHL (*MYC + BCL2* &/or *BCL6* rearrangement). Also, 20 patients (11.6%) showed a concurrent expression of *BCL2* and *MYC* oncoproteins (DEL) on IHC. Most (6/7) DHL patients were double expressors also. The DHL patients demonstrated a significant association with female gender, high serum LDH levels (> 750 U/L) and GCB phenotype. DEL patients contrarily predominated amongst males, had intermediate LDH levels (251–500 U/L) and non GCB phenotype. The OS of the patients was 63.8% at 4 years. The OS of the DLBCL, DEL and DHL patients was 71.9%, 46.9%, and 0%, respectively at 4 years (p value 0.010). In case of DEL subtype, factors such as age < 60 years (66.7%), male sex (60.8%), nodal localization (52.5%), early disease stage (84.6%), low IPI score (60%), absence of B symptoms (50%), LDH < 250 U/L (80%) and GCB phenotype (53.3%) were associated with better OS. Further, the OS of DHL cases was 0% at 4 years. Double hit and double expresser lymphomas have poor prognostic outcomes and should be separated from DLBCL. All DELs should be tested for DHLs and especially those with immunoblastic morphology. DHL and DEL subtypes delineate the subtypes with inferior OS and reinstate the need for aggressive interventions.

Keywords Double hit lymphoma · Double expresser lymphoma · Diffuse large B cell lymphoma · C-MYC · BCL2 · BCL6

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Introduction

Current therapeutic decisions on the treatment of lymphomas are based upon the histological classification. Diffuse large B cell lymphomas (DLBCL) account for nearly 30% of all lymphomas with diverse behaviour and therapeutic outcomes [1–3]. Gene expression profiling (GEP) of nearly 20,000 genes has identified various

subtypes of DLBCL namely Germinal Centre 'B' Cell (GCB) type, Activated 'B' Cell (ABC) type and Mediastinal 'B' Cell type which also show differences in their pathogenetic mechanisms [4–6]. This Cell of Origin (COO) based classification has emphatically separated the survival in favour of GCB type. Yet, around 20% patients of GCB type recur within the first year of therapy while 50% of cases of ABC type have good long term survival [6, 7]. The COO classification of DLBCL therefore is inadequate to prognosticate effectively.

Further research into DLBCL has helped define the occult categories within DLBCL based upon *MYC* oncogene rearrangement either alone or in combination with *BCL2* &/or *BCL6* and are referred to as single hit, double hit or triple hit lymphomas (SHL/DHL/THL) [8–10]. These subtypes have been identified in 2–12% of DLBCL patients with poor response to standard R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone) therapy [9–13]. An advanced stage of the disease, high International Prognostic Index (IPI), markedly raised lactic acid dehydrogenase (LDH), bone marrow involvement and early disease progression have also been associated with DHL [9–17]. Researchers attempting to separate these cases by immunohistochemistry (IHC) as surrogates [18, 19] serendipitously found a new group of 'Double Expresser' lymphomas (DEL) that showed concurrent expression of *MYC* and *BCL2* oncoprotein but were not necessarily rearranged at the genetic level to always qualify as DHL [19–22].

The present study was conducted with the aim to identify the DHL/DEL patients amongst the DLBCL cases so as to separate these subsets on the basis of their distinctive clinicopathological profile and overall survival (OS) with the ultimate goal of enriching the cohort for genetic rearrangement testing for the identification of DHL patients (testing for all cases of DLBCL will not be cost effective or readily available).

Materials and Methods

The present study is a retrospective analysis involving 172 cases of DLBCL diagnosed between January 2014 and December 2015 at a tertiary cancer care centre. These cases were retrieved from the archives and re-examined for confirmation of diagnosis of DLBCL.

Initially, 200 cases of DLBCL were selected for the study, out of which, 28 cases of core biopsies were found to be inadequate for complete workup and were excluded leaving a total of 172 evaluable cases. For 120 (67.9%) cases, core biopsy was the available tissue while 52 cases (30.3%) had excision biopsy. The cases were then further sub-classified on the basis of cell of origin (COO) into

GCB and Non-GCB type using Hans algorithm [23]. In addition, all the cases were further immunostained for MYC and BCL2 to identify the DEL subtype. Immunostaining for Ki-67 was also performed in all the cases. IHC was performed on Ventana Benchmark XT, Tucson, Arizona, USA employing heat induced epitope retrieval and ultra view labelling system.

For immunohistochemical analysis, the percentage of positive cells and their mean intensity of staining was recorded. The panel of markers is outlined in Supplementary Table 1. Threshold percentage for assessing positivity was moderate to strong nuclear staining in $\geq$ 40% of the cells for MYC, strong nuclear staining in $\geq$ 30% of the cells for BCL6 & MUM1, strong cytoplasmic staining in $\geq$ 70% of the cells for BCL2 and strong membranous staining in $\geq$ 30% of the cells for CD10 respectively [19–22, 24]. Any intensity of nuclear staining with Ki-67 antibody was considered positive and contributed to Ki-67 labelling index [25].

The rearrangement studies were performed using break apart Fluorescent in situ hybridization (FISH) with standard probes procured from ZytoVision. The panel of FISH probes is given in Supplementary Table 2. The test was performed on 4-micron formalin fixed and paraffin embedded tissue sections following standardized protocol which included pre-treatment (dewax/proteolysis), denaturation, probe application and hybridization, application of DAPI/antifade solution and analysis of slides using Leica fluorescent microscope (DM6000B). At least 100 contiguous non-overlapping nuclei were assessed and percentage of positive nuclei was computed. Cases with break apart signals in greater than/equal to 15% of nuclei (laboratory determined cut off based upon presence of split signals in reactive lymph nodes) were considered positive for presence of rearrangement. Figure 1 shows a representative staining of DEL on IHC and DHL by FISH.

SPSS version 23 for Windows (SPSS Inc, Chicago IL, USA) was used for statistical analysis. Pearson χ^2 or Fisher's Exact Test, whichever appropriate, was used for categorical variables. OS analysis was performed using the Kaplan–Meier method [26]. OS was calculated as the time from the date of initial diagnosis to the date of death or the date of last follow-up. Log Rank test was used to compare the difference in survival among the groups. A two sided p value < 0.05 was considered as significant. This study was approved by the Institutional Review Board of the Institute (IRB No. RGCIRC/IRB/59/2016) and was performed in accordance with the principles of the Declaration of Helsinki.
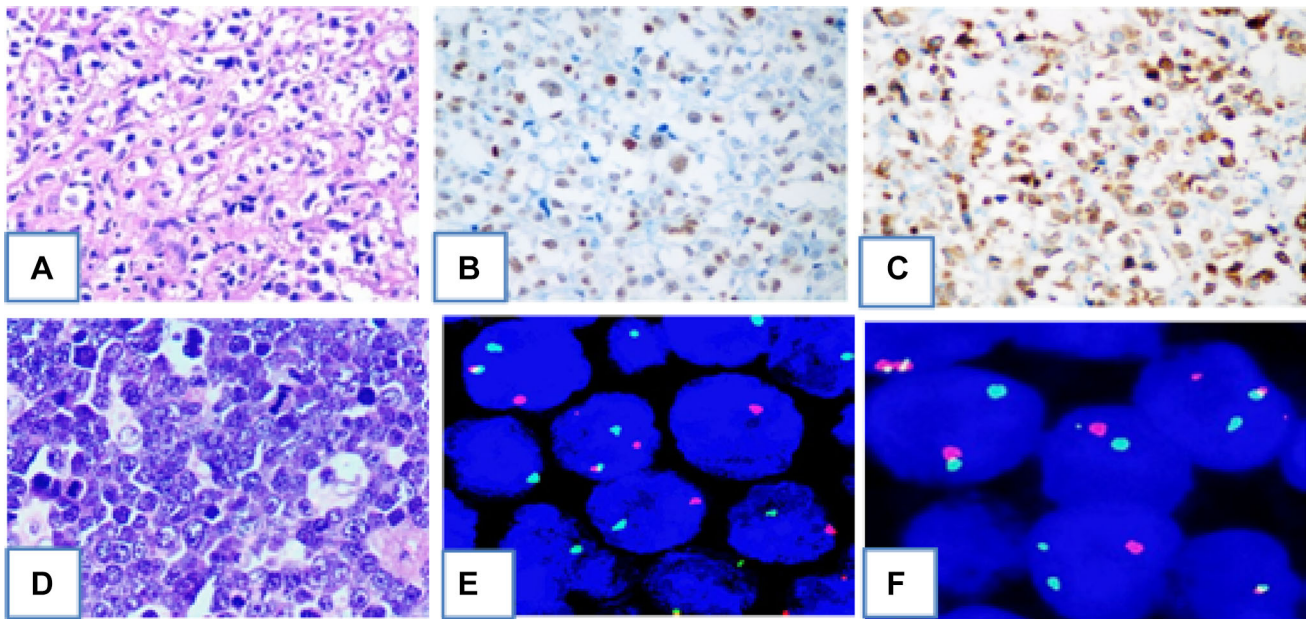
Fig. 1 Representative images of a case of double expresser (**a**) H&E stained section, (**b**) CMYC positivity (> 40% nuclear staining) on IHC (**c**), BCL2 positivity (> 70% cytoplasmic staining) on IHC (**c**); **d**–**f** a case of double hit lymphoma (**d**) H&E section which showed BCLU morphology, (**e**) C-MYC rearrangement on FISH using break apart probe seen as separate red and green signals, (**f**) BCL6 rearrangement on FISH using break apart probe seen as separate red and green signals

Results

A total of 172 patients with a diagnosis of DLBCL were included in the study of which 57.6% were males. The mean age of the patients was 56 years. As per the classification based on COO, 49.4% patients were GCB type. The demographic, clinical and pathological profile of the patients is presented in Table 1. Majority of the patients presented with stage III–IV disease (60.5%), extranodal site (57%), IPI score 0–2 (50.6%), presence of B symptoms (52.9%), LDH levels 251–500 U/L (39%), centroblastic morphology (86.1%), ABC phenotype (50.6%) and rituximab based regime (87.2%).

Table 2 shows the comparison of demographic, clinical and pathological parameters of DLBCL, DHL and DEL cases. Seven cases of DHL were identified on rearrangement study (MYC + BCL2-4/7; MYC + BCL6-2/7; MYC + BCL2 + BCL6-1/7). The DHL patients were more commonly associated with age group ≥ 60 years (57.1%), female sex (85.7%), stage III–IV disease (100%), extranodal site (71.4%), IPI score 3–5 (100%), presence of B symptoms (100%), LDH levels > 750 U/L (100%) and GCB phenotype (100%). The Ki-index of the DHL cases ranged from 50 to 95% (mean 80 ± 15.5). The mean index was 69.8 ± 15.4 in the DLBCL cases (range 30–99).

Twenty patients (11.6%) of DEL with a concurrent expression of BCL2 and MYC oncoproteins (DEL) on IHC were identified. Among these, 14 (70%) patients were of non GCB phenotype whereas, 6 (30%) patients were of

Table 1 Demographic, clinical and pathological profile of 172 patients with diffuse large B-cell lymphoma

Characteristics	N (%)
Age (years)	
< 60/≥ 60	86 (50)/86 (50)
Sex	
Female/male	73 (42.4)/99 (57.6)
Stage	
I–II/III–IV	68 (39.5)/104 (60.5)
Site	
Extranodal/nodal	98 (57)/74 (43)
IPI score	
Low (0–2)/high(3–5)	87 (50.6)/85 (49.4)
B symptoms	
Present/absent	91 (52.9)/81 (47.1)
LDH (U/L)	
< 250/251–500/501–750/> 750	57 (33.1)/67 (39)/23 (13.4)/25 (14.5)
Morphology	
Centroblastic/immunoblastic/ anaplastic/BCLU	148 (86.1)/12 (7)/6 (3.5)/6 (3.5)
Phenotype	
GCB/non GCB	85 (49.4)/87 (50.6)
Treatment regime	
Rituximab based/non rituximab based/no treatment	150 (87.2)/7 (4.1)/15 (8.7)

N number, *IPI* International Prognostic Index, *LDH* lactic acid dehydrogenase, *BCLU* B cell lymphoma unclassifiable, *GCB* germinal centre type

Table 2 Comparison of demographic, clinical and pathological parameters of DLBCL, DHL and DEL cases

| Characteristics	DLBCL n (%)	DHL n (%)	DEL n (%)	p value*	p value**	p value***
N	145	7	20	–	–	–
Age (years)						
< 60	75 (51.7)	3 (42.9)	8 (40)	1.000	0.341	1.000
≥ 60	70 (48.3)	4 (57.1)	12 (60)			
Sex						
Male	86 (59.3)	1 (14.3)	12 (60)	**0.042**	1.000	**0.041**
Female	59 (40.7)	6 (85.7)	8 (40)			
Stage						
I–II	61 (42.1)	0 (0)	7 (35)	**0.042**	1.000	**0.025**
III–IV	84 (57.9)	7 (100)	13 (65)			
Site						
Extranodal	83 (57.2)	5 (71.4)	10 (50)	0.699	0.633	0.408
Nodal	62 (42.8)	2 (28.6)	10 (50)			
IPI score						
Low (0–2)	79 (54.5)	0 (0)	8 (40)	**0.005**	0.242	**0.015**
High (3–5)	66 (45.5)	7 (100)	12 (60)			
B symptoms						
Present	72 (49.7)	7 (100)	12 (60)	**0.014**	0.477	0.068
Absent	73 (50.3)	0 (0)	8 (40)			
LDH (U/L)						
< 250	52 (35.9)	0 (0)	5 (25)	**< 0.0001**	0.182	**0.004**
251–500	61 (42.1)	0 (0)	6 (30)			
501–750	18 (12.4)	0 (0)	5 (25)			
> 750	14 (9.7)	7 (100)	4 (20)			
Morphology						
Centroblastic	131 (90.3)	5 (71.4)	12 (60)	0.121	**< 0.0001**	0.908
Immunoblastic	6 (4.1)	1 (14.3)	5 (25)			
Anaplastic	5 (3.5)	0 (0)	1 (5)			
BCLU	3 (2.1)	1 (14.3)	2 (10)			
Phenotype						
GCB	72 (49.7)	7 (100)	6 (30)	**0.014**	0.099	**0.001**
Non GCB	73 (50.3)	0 (0)	14 (70)			
Treatment regime						
Rituximab based	126 (86.9)	7 (100)	17 (85)	1.000	**0.023**	0.545
Non Rituximab based	4 (2.8)	0 (0)	3 (15)			
No treatment	15 (10.3)	0 (0)	0 (0)			

The figures in bold indicate significant associations

N, number; LDH, lactic acid dehydrogenase; IPI, International Prognostic Index; BCLU, B cell lymphoma unclassifiable; GCB, germinal centre type; DLBCL, diffuse large B-cell lymphoma; DHL, double hit lymphomas; DEL, double expresser lymphomas; p value*, DLBCL versus DHL; p value**, DLBCL versus DEL; p value***, DHL versus DEL

GCB phenotype. DEL patients showed a predominance of characteristics such as age group ≥ 60 years (60%), male sex (60%), stage III–IV disease (65%), IPI score 3–5 (60%), presence of B symptoms (60%), LDH levels 251–500 U/L (30%) and non GCB phenotype (70%). None of the features significantly associated with DHL was repeated in DEL (Table 2). The Ki-index of the DEL cases ranged from 50 to 100% (mean 81.7 ± 13.7). Immunoblastic morphology over represented significantly (25%) in DEL.

Six of seven DHL patients were double expressors as well. One case of DHL which was not DEL showed 0% and 75% immunostaining for *MYC* and *BCL2* oncoproteins, respectively with a Ki-index of 90%.
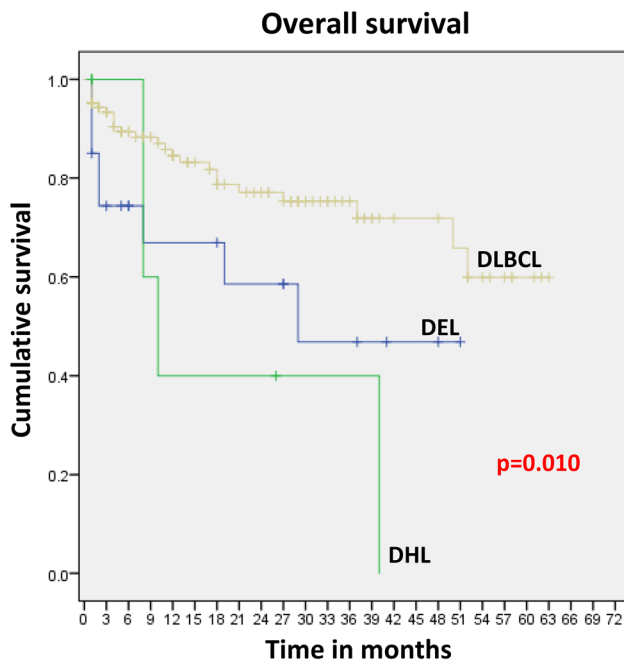
Overall survival

Fig. 2 Overall survival of DLBCL, DHL and DEL cases. Comparison of OS between DLBCL versus DHL, *p* value 0.010; DLBCL versus DEL, *p* value 0.036; DEL versus DHL, *p* value 0.546. *DLBCL* diffuse large B-cell lymphoma, *DHL* double hit lymphomas, *DEL* double expresser lymphomas

The OS of the patients was 63.8% at 4 years. Figure 2 profiles the OS of the DLBCL, DEL and DHL patients which was 71.9%, 46.9%, and 0%, respectively at 4 years (*p* value 0.010). In case of DEL cases, factors including age < 60 years (66.7%), male sex (60.8%), nodal site of disease (52.5%), early disease stage (84.6%), low IPI score (60%), absence of B symptoms (50%), LDH < 250 U/L (80%) and GCB phenotype (53.3%) were associated with better OS. However, variations were noted in the case of DHL patients and all the 7 patients with a diagnosis of DHL had died at 4 years and hence the OS was 0% with respect to the various factors.

Incidentally, two cases were seropositive for HIV, of which, one patient had stage I DLBCL while the other had stage III DLBCL with plasmablastic differentiation. None of these two cases were DEL or DHL. Leukemic phase was seen in four cases of DLBCL of which one was a case of DEL.

Discussion

DLBCL is considered a heterogenous and an aggressive lymphoma with varied clinical outcome [9, 15]. Attempt to prognostically categorise these lymphomas according to COO led to the identification of GCB and non GCB phenotype. In our study, majority of the patients were non GCB type (50.6%) which is in agreement with the study by Ayurek et al. [27]. However, our results are dissimilar when compared to the study by Johnson et al. [18] and Hu et al. [21] who reported a higher percentage of GCB cases in their cohort (76% and 66%, respectively).

The incidence of DHL in our study was 4%, which is comparable to the study by Scott et al. [11], Barrans et al. [28], Visco et al. [29]. Six of seven DHL patients were females which is in stark contrast to most studies which state that DHL cases are more common in males [17, 30]. All cases of DHL had stage III/IV disease and high LDH levels. Most cases had extranodal disease. Bone marrow and CNS involvement was seen in 66.6% and 33.3% cases, respectively. The clinicopathological parameters were similar to that reported in the previous studies [2, 4, 9–20, 28–33]. The authors recommend that these unique clinical features can be used to select the patients of DLBCL for further testing for molecular cytogenetics for rearrangement.

DEL are defined as lymphomas having concurrent expression of MYC and BCL2 oncoprotein. It was initially thought that DEL are same as DHL, however, subsequent studies revealed that DEL are not equivalent to DHL, even though 80–90% of the DHL are also DEL. Apart from gene rearrangement (which defines DHL cases), gene can be amplified or mutated which result in increased oncoprotein expression (which define DEL cases) [9, 22, 24]. In our study, there were 20 (11.6%) cases of DEL. The incidence is slightly lower than stated in the literature (19–34%) [22, 24, 34]. Interestingly, 6 out of 7 DHL patients were also DEL. Since DEL concentrates DHL cases, all cases of DEL should undergo FISH testing for assignment as DHL. Moreover, such separation is also necessary as DEL cases which were not DHL fared better than the former. This observation is a reaffirmation of the studies by Hu et al. and Green et al. [20, 22, 24].

In our study, the Ki-index of DHL ranged from 50 to 95%, whereas that of DEL cases ranged from 50 to 100%. This observation brings forth the fact that Ki-index cannot be relied upon to sift out these subsets of lymphomas from the DLBCL cases in contrast to the commonly held belief that DHL and DEL are highly proliferative lymphomas with Ki67 labelling exceeding 95% [9, 35, 36]. Our observation, however, resonates with the study by Mationg-Kalaw which also highlighted the lack of value of Ki67 in enriching the DLBCL cases for genetic rearrangement studies [37]. We propose a schema (Fig. 3) for the assignment of DEL and DHL subtypes based upon our findings with a view to optimize the use of resources.

The OS of the entire cohort was 63.8% at 4 years and was 71.9%, 46.9%, and 0% at 4 years for DLBCL, DEL and DHL respectively. In DEL cases, factors like age < 60 years, male sex, nodal site of disease, early disease
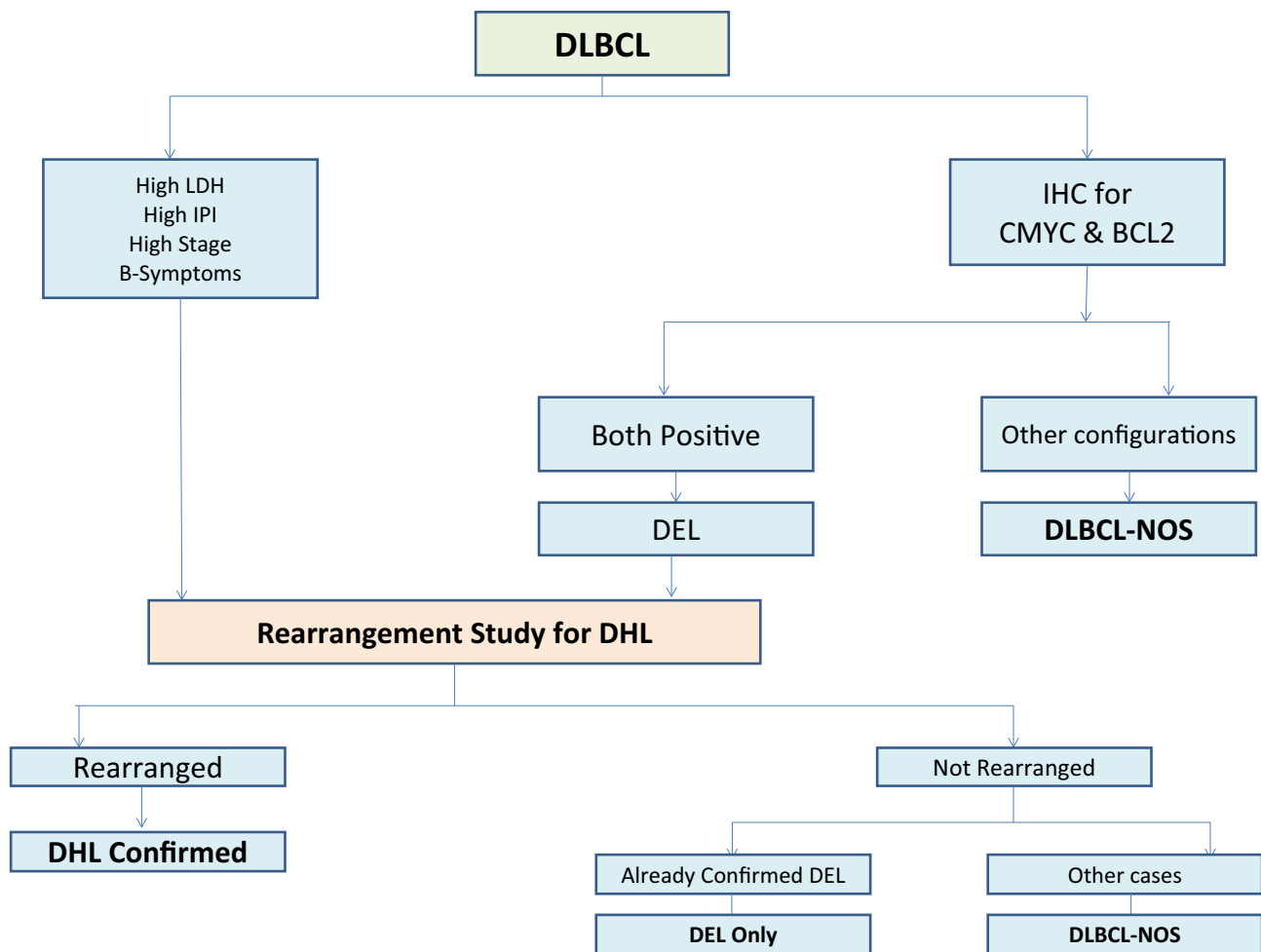
Fig. 3 Proposed algorithm for the testing of DHL/DEL patients. *DLBCL* diffuse large B-cell lymphoma, *DHL* double hit lymphomas, *DEL* double expresser lymphomas

stage, low IPI score, absence of B symptoms, LDH < 250 U/L and GCB phenotype were associated with better OS. However, all 7 DHL patients, irrespective of the afore mentioned factors that tempered the outcome in DEL died by the end of 4 years of follow up. The statistical correlations observed in terms of OS among the different groups points towards the fact that the specific subtypes of DLBCL patients considered along with clinico-pathological factors determines the survival. The 4-year OS in patients with DEL was 56% (95% CI 40–69%). In a study by Herrera et al., the 4 year OS in patients with DHL was inferior to that of patients without DHL (25% vs. 66%; $p < .001$). Patients with DHL had decreased OS (25%; 95% CI 5–54%) compared with patients with DEL but not DHL (OS 61%, 95% CI 45–74%), and patients with neither DEL nor DHL (OS 70%, 95% CI 55–80%; P = .002) [38].

The strength of the present study is the comprehensive IHC and molecular diagnostic work up that allowed definitive diagnosis of DHL/DEL patients and study their distinctive clinicopathological profile and OS. These findings will help delineate a smaller cohort of patients that shall undergo genetic rearrangement testing to select patients with DHL/DEL. The limitations of the study include the fact that some previously stored biopsies were inadequate for performing the IHC and rearrangement studies, thus compromising on the sample size. Multivariate analysis was not performed and the COO was identified by surrogate IHC with its inherent discordance with gene expression profiling in 10–15% of the cases.

In conclusion, the double hit and double expresser lymphomas have poor prognostic outcomes. There are significant differences between DLBCL and DHL in stage at presentation, IPI, LDH levels, B symptoms, phenotype, and morphology and these clinicopathological parameters can enrich the subset of population for testing for *MYC*, *BCL2* and *BCL6* rearrangements using FISH. Such distinctive features were however not observed in the DEL group. All patients of DLBCL therefore must undergo

additional *MYC* and *BCL2 IHC* to identify DEL. DELs however, concentrate DHLs to the extent of 85% and hence once identified, all DELs should be tested for DHLs. DHL and DEL subtypes delineate the subtypes with inferior OS and reinstate the need for aggressive interventions.

Compliance with Ethical Standards

Conflict of interest None.

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