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"A Retro-Prospective Observational Study to Determine the Proportion of Double Expressors in Diffuse Large B-Cell Lymphomas, Not Otherwise Specified (DLBCL, NOS) in a Tertiary Care Setting."

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Abstract

"A retro-prospective observational study to determine the proportion of double expressors in diffuse large b-cell lymphomas, not otherwise specified (dlbcl, nos) in a tertiary care setting."

Aim: *To study the proportion of cases of Double Expressors (DE) in Diffuse Large B-Cell Lymphomas, not otherwise specified (DLBCL, NOS) in a tertiary care setting.*

Objectives

1. To determine the proportion of cases of Double Expressors which show coexpression of BCL-2 and c-myc proteins by Immunohistochemistry in cases of DLBCL, NOS.

2. To characterize the patients according to their age-groups, gender and transformation from a lower grade lymphoma and to report these numbers.

3. To correlate, when possible, with the results of Fluorescent In-Situ Hybridization (FISH) to detect cases that harbor a c-myc translocation also showing a BCL2 translocation therefore belonging in the category of Double- Hit (DH) lymphoma.

4. To associate between the diagnosis of DE status and the clinical outcome of the patient at a minimum follow-up period of 1-year post diagnosis (regression of primary tumor, appearance of new lesions and CNS metastases or death.)

Materials and Methods: *Specimens from nodal and extra-nodal sites diagnosed as Diffuse Large B-Cell Lymphoma, not otherwise specified (DLBCL, NOS) preserved as paraffin blocks were subjected to Immunohistochemical (IHC) testing for determination of c-myc and BCL2 protein expression. The data was collected from the Amrita HIS and MRD viewer. In co-ordination with the clinical co-guide, the outcome of the patients at a minimum follow-up of 1-year post diagnosis was documented in terms of clinical status and imaging.*

Observations & Results: *85/127 cases were noted to be Double Expressors and represented 66.9% of the total cases and 32/127 were noted to be Triple Expressors represented 25.1% of the total cases of DLBCL, NOS.*

Using the Hans algorithm, it was noted that 66 cases were of the GCB phenotype (66%) and 34 cases were of the ABC phenotype (34%.) Double Expressors (78.78%) and Triple Expressors (31.81%) were seen more commonly in the cases with GCB phenotype. 8 cases of transformations from low grade non-Hodgkin's lymphoma were noted and 4 (50%) were known cases of Follicular lymphoma. 16 cases underwent testing by FISH. 7/16 (43.7%) cases were Double Expressors for BCL2 and c-myc by IHC. 2/7 (28.5%) cases were positive for a genetic aberration of c-myc gene, and were thus considered to be Double Hit Lymphomas. In our study, 19% of the total cases of DLBCL, NOS was the proposed population proportion of Double Hit Lymphomas. The clinical evaluation of patients at 1 year post diagnosis revealed that DLBCL, NOS cases of the GCB phenotype showed a better response to chemotherapy and had a better prognosis, as compared to their ABC phenotype counterparts. Post chemotherapy, the highest rate of relapse of 25% was seen in the group showing strong >70% staining for c- myc.

Conclusion

The results are reliable enough to predict the proportion of Double Expressors and Triple Expressors among total number of DLBCL, NOS cases. These cases should ideally be investigated with the help of FISH or PCR for detection of genetic aberrations. This study also predicts the proportion of Double Hit Lymphomas among Double Expressor Lymphomas.

Cases of DLBCL, NOS should also be screened according to the intensity of c-myc staining by IHC. The number of cases with genetic aberrations may not always meet the international reporting guidelines for screening for protein over-expression (40% nuclear positivity for c-myc.) The guidelines for reporting c-myc positivity by IHC should be reviewed to include a greater range of subjects. Cases showing c-myc staining > 70% positivity should be screened for CNS lesions to rule out involvement or relapse at the time of diagnosis and regular follow-up visits. If and when resources permit, all cases of DLBCL, NOS can be evaluated for genetic aberrations so that the ideal targeted chemotherapy is received by the patient to improve prognosis.

List of Abbreviations

ABC : Activated B-Cell

ASCT : Autologous Stem Cell Transplant

BCL2 : B-Cell lymphoma 2 protein

BCL6 : B-Cell lymphoma 6 protein

CAR-T cell : Chimeric Antigen Receptor T-Cell

CD : Cluster of Differentiation

CHOP : Cyclophosphamide, Hydroxyduanorubicin, Vincristine Sulphate, Prednisone

c-myc : Cellular myelocytomatosis oncogene

COO : Cell of Origin

CORAL: Collaborative Trial in Relapsed Aggressive Lymphoma

CNS : Central Nervous System

CSF : Cerebrospinal fluid

CNS-IPI: Central Nervous System International Prognostic Index

CT : Computed Tomography

DAB : Diaminobenzidine tetrahydrochloride

DE : Double Expressor

DEL : Double Expressor Lymphoma

DH : Double Hit

DHL : Double Hit Lymphoma

DLBCL: Diffuse Large B-Cell Lymphoma

EBV : Epstein-Barr Virus

EPOCH: Etoposide, Prednisone, Oncovin, Cyclophosphamide, Hydroxyduanorubicin

FDG-PET: Fluorodeoxyglucose Positron Emission Tomography

FNAB : Fine Needle Aspiration Biopsy

FISH : Fluorescent in-Situ Hybridization

GCB : Germinal Centre B-Cell

GEP : Gene Expression Profiling

H&E : Hematoxyline & Eosin

IHC : Immunohistochemistry

IRF4 : Interferon regulatory factor 4

IPI : International Prognostic Index

LDH : Lactate dehydrogenase

MUM1 : Multiple Myeloma Oncogene 1

MRD : Medical records department

c-myc : c-myc oncogene

NCCN : National Comprehensive Cancer Network

NHL : non-Hodgkin's Lymphoma

NOS : Not otherwise specified

OS : Overall survival

PAX : Paired box family of transcription factors

PCR : Polymerase chain reaction

PET : Positron Emission Tomography

PFS : Progression-free survival

RT-PCR : Real Time Polymerase chain reaction

R-hyper CVAD: Hyperfractionated Cyclophosphamide, Vincristine, Doxorubicin, & Dexamethasone alternating with Methotrexate and Cytarabine

R-CODOX-M/IVAC: Cyclophosphamide, Vincristine, Doxorubicin, and high-dose Methotrexate alternating with Ifosfamide, Etoposide, and Cytarabine

R-C HOP : Rituximab, Cyclophosphamide, Hydroxyduanorubicin, Oncovin, Prednisone

R-D HAP : Rituximab, Dexamethasone, High-Dose Cytarabine, and Cisplatin

R-E POCH : Rituximab, Etoposide, Prednisone, Oncovin, Cyclophosphamide Hydroxyduanorubicin

R-ICE : Rituximab, Ifosfamide, Carboplatin, and Etoposide

SD : Standard deviation

SPSS : Software Package for the Social Sciences

TE : Triple Expressor

TEL : Triple Expressor Lymphoma

WHO : World Health Organization

Introduction

The Rappaport Classification system for lymphomas was introduced in 1958. At that time, it was thought that large cell tumors were not of lymphoid origin. The system highlighted the origin of certain large cell tumors. Treatment was mainly palliative and consisted of nitrogen mustard and anti-metabolites combined with radiation therapy. In the 1980s there was an introduction of a new classification for lymphomas based on clinical course of the disease along with the use of complex combination therapies. The CHOP regimen changed the treatment landscape for Non-Hodgkin's Lymphomas. Nowadays the WHO classification with over 40 subtypes of Lymphomas exists and with it came a more focused and dedicated approach to clinical research and better drugs. The monoclonal antibody Rituximab showed efficacy in the treatment of NHL in combination with other modalities of chemotherapy, but today, 15 years later this efficacy has been rivalled and exceeded.

Diffuse Large B-Cell Lymphoma (DLBCL) is a clinically and biologically heterogenous disease. DLBCL, NOS constitutes 25-35% of adult Non-Hodgkin Lymphomas in developed countries and higher, in developing countries.[40] DLBCL is a neoplasm of medium or large B lymphoid cells whose nuclei are the same size as, or larger than, those of normal macrophages, or more than twice the size

of those of normal lymphocytes, with a diffuse growth pattern. Morphological, biological, and clinical studies divide DLBCLs into morphological variants, molecular sub-types, and distinct disease entities. However, there remain many cases that may be biologically heterogeneous which are classified as DLBCL, NOS, which encompasses all cases that do not belong to a specific diagnostic category listed.

Table No. 1: Classification of Large B-cell lymphomas

WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues, 2017 Revised

Diffuse large B-cell lymphoma, NOS
Common morphologic variants
Centroblastic
Immunoblastic
Anaplastic
Rare morphologic variants
Molecular subgroups
GCB-like
ABC-like
Immunohistochemical subgroups
CD5-positive DLBCL
GCB-like
Non-GCB-like
Diffuse large B-cell lymphoma subtypes/entities
Primary mediastinal (thymic) large B-cell lymphoma
T-cell/histiocyte-rich large B-cell lymphoma
Intravascular large B-cell lymphoma
Primary DLBCL of the CNS
Primary cutaneous DLBCL, leg type
DLBCL associated with chronic inflammation
ALK-positive DLBCL
Large B-cell arising in HHV8-associated multicentric Castleman disease
Plasmablastic lymphoma and primary effusion
Borderline cases
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin's lymphoma

GCB: Germinal-center B-cell, ABC: Activated B-cell, NOS: Not otherwise specified, ALK: Anaplastic lymphoma kinase, DLBCL: Diffuse large B-cell lymphoma

DLBCL, NOS can be subdivided into germinal centre B-cell (GCB) subtype and activated B-cell (ABC) subtype. c-myc protein is expressed in 30-50% of diffuse large B cell lymphoma (DLBCL) and is associated with concomitant expression of BCL2 in 20% to 35% of cases. DLBCLs with co-expression of c-myc and BCL2 are called double-expressor lymphomas (DELs). There is an overexpression of the c- myc oncogene and BCL2 (≥40% and >50% positive staining by IHC, respectively); whereas double-hit lymphomas (DHLs) have c-myc and BCL2 or BCL6 rearrangement as detected by fluorescence in situ hybridization (FISH) or standard cytogenetics. c-myc/BCL2 double expression is an independent risk factor of DLBCL relapse or progression.

c-myc rearrangement (c-myc+) and DH/THL remains significantly prognostic at the time of relapse. DELs account for approximately one-third of de novo disease and up to 50% of relapsed/refractory (RR) DLBCL. In several studies, DELs were shown to have worse outcomes than other DLBCLs. Poor response to standard chemotherapy CHOP or R-CHOP is seen with DHLs and DELs with a median overall survival of <12 months. To corroborate this, previous studies have shown that when comparing cases treatment with R-CHOP and with R-EPOCH, patients treated with R-EPOCH had a better clinical outcome in terms of median overall survival (OS).[38]

Etiology

These tumours usually arise de novo(primary) but can also represent transformation of a less aggressive lymphoma (secondary), such as chronic lymphocytic leukaemia/ small lymphocytic lymphoma, follicular lymphoma, marginal zone lymphoma, or nodular lymphocyte predominant Hodgkin lymphoma. DLBCLs, NOS, occurring in the setting of immunodeficiency are more often EBV-positive than sporadic cases.

Tumor localization

Patients present with nodal or extranodal disease. The most commonly involved extranodal site is the gastrointestinal tract (stomach and ileocaecal region; Other common sites include bone, testes, spleen, Waldeyer ring, salivary glands, thyroid, liver, kidneys, and adrenal glands. Bone marrow involvement in DLBCL can be discordant (low-grade B-cell lymphoma in the marrow, seen in 10- 25% of cases) or concordant (large cell lymphoma in the marrow.) Studies have suggested that FDG- PET is a sensitive technique for detecting concordant bone marrow involvement but is not reliable for discordant disease. The most recent consensus criteria for lymphoma staging indicate that a routine staging bone marrow biopsy is no longer required if FDG-PET is negative.

Morphologic involvement of the peripheral blood by DLBCL is rare. Staging investigations include CT scan with or without PET/CT scan, bone marrow aspirate, and biopsy. Limited stage is defined as stage 1A/2A with non-bulky (<10 cm) disease. CNS-directed investigations, Clinical features There is usually a rapidly enlarging tumour mass at single or multiple nodal or extranodal sites at presentation. Almost half of the patients have stage I or II disease, but the inclusion of PET/CT in the initial staging of DLBCL has resulted in stage migration.

Microscopy

Three common and several rare morphological variants have been recognized: Centroblastic, Immunoblastic and Anaplastic.

Immunophenotype

The neoplastic cells typically express pan-B-cell markers such as CD19, CD20, CD22, CD79a, and PAX5. The expression of c-myc and BCL2 varies considerably; In most studies, BCL2 is considered positive if 50% of the tumor cells are positive, and c-myc is considered positive if 40% of the tumor cell nuclei are positive. Co- expression of these two proteins (so-called double-expressors) is more frequent in the ABC subtype.

The Hans algorithm uses three markers to distinguish the GCB from the non-GCB subtype: CD10, BCL6, and IRF4/MUM1 and are each considered positive if 30% of the tumor cells stain positively. CD10 is positive in 30-50% of cases, BCL6 in 60- 90%, and IRF4/MUM1 in 35-65% cases. In normal GCBs expression of IRF4/ MUM1 and BCL6 is mutually exclusive, whereas coexpression of these markers is found in 50% of DLBCLs. BCL2 expression is closely linked to the presence of t (14;18) (q32; q21.3) and is more common in the ABC subtype, but is the result of copy number gains and transcriptional upregulation.

Cell of origin/ postulated normal counterpart

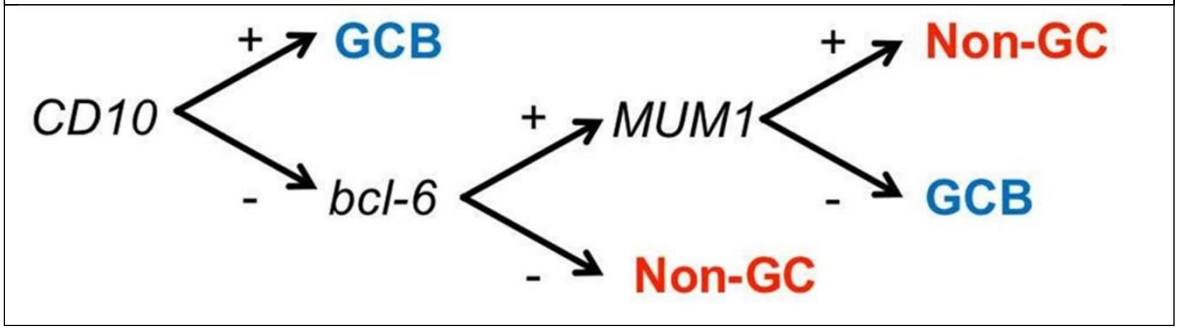
The postulated normal counterparts are peripheral mature B cells of either germinal centre origin (GCB subtype) or germinal centre exit I early plasmablastic or post- germinal centre origin (ABC sub-type), which are associated with survival differences in patients treated with the CHOP chemotherapy regimen plus rituximab (R-CHOP).

Eligibility in newer clinical trials requires the determination of cell of origin status because preliminary data from phase I/II trials suggest that the benefit from the addition of bortezomib, lenalidomide, and ibrutinib to R-CHOP is preferentially seen in the ABC subtype. Therefore, the distinction between the GCB subtype and

the ABC subtype should be made for all cases of DLBCL, NOS, at diagnosis. If gene expression technologies are not available, immunohistochemistry technologies are considered an acceptable alternative. The algorithm used should be specified.

The Hans algorithm (also Tally) uses three markers to distinguish between them in clinical practice:

Figure 1: Hans Algorithm



Genetic profiling

Chromosomal translocations: 30% of cases show re-arrangement of the 3q27 region involving BCL6, which is the most common translocation in DLBCL and occur commonly in the ABC subtype. Translocation of the BCL2 gene occurs in 20-30% of DLBCL cases commonly in the GCB subtype. It is present in about 40% of cases and is closely associated with BCL2 and CD10 protein expression. c-myc rearrangement is observed in 8-14% of cases evenly distributed between the GCB subtype and the ABC subtype; half of the DLBCL cases that harbour a c-myc translocation also show a BCL2 and/or BCL6 translocation, (Double-Hit Lymphoma.) Cases with typical DLBCL morphology and isolated c-myc translocation belong in the category of DLBCL, NOS. Most DLBCL, NOS cases with a c-myc translocation are also Double Expressers (i.e are positive for both c-myc and BCL2 protein.)

Prognostic and predictive factors

Clinical features: The 5-year progression-free and overall survival rates are about 60% and 65%, respectively. Stage and patient age are factors that affect survival. The International Prognostic Index (IPI) incorporates five clinical variables including age, lactate dehydrogenase (LDH), number of extranodal sites, Ann Arbor stage, and Eastern Cooperative Oncology Group (ECOG) performance status were used to risk stratify and identify 4 discrete risk categories. [39] Other prognostic factors associated with inferior outcome include tumour bulk (~ 10 cm), male sex, vitamin D deficiency, low body mass index, elevated serum free light chains, monoclonal serum lgM proteins, low absolute lymphocyte/monocyte count, and concordant (but not discordant) bone marrow involvement. A study published by Zhou Z, Sehn LH, Rademaker AW, Gordon LI, LaCasce AS, Crosby-Thompson A et. al. in Blood concluded that the National Comprehensive Cancer Network (NCCN) IPI is a robust and

useful tool to stratify prognostically relevant subgroups of DLBCL patients in the current era of rituximab-based therapy. Compared with the IPI and other modifications of the IPI, it better incorporates 2 known continuous prognostic variables, age and LDH, in a rational way that is both simple to apply and valid in the rituximab era. With its enhanced capacity to discriminate risk groups, it has value in treatment planning and in discussions of prognosis. Its utility can also be found in stratification of future randomized clinical trials. Because there is continued enthusiasm for defining a high-risk group in the R-CHOP era, the NCCN-IPI will be useful in identifying candidates for novel approaches including in post remission therapies such as intensification with autologous stem cell transplant or consolidation/maintenance with new targeted agents. [37] Immunophenotyping: BCL2 and BCL6 are biomarkers of which the reported prognostic effect was altered by the addition of Rituximab to the CHOP chemotherapy regimen. Predictive markers include those for determination of Cell of Origin (i.e GCB subtype vs ABC subtype) being tested for phase III clinical trials and markers of the presence of relevant oncogene translocations. The double-expression of c-myc and BCL2 proteins is found in approximately 30% of all cases of DLBCL, NOS and is associated with inferior survival according to studies. Double-expression status also predicts increased risk of CNS relapse in DLBCL, NOS, and is independent of the CNS International Prognostic Index (CNS-IPI.)

Genetics: The presence of a BCL2 translocation is associated with inferior outcome in GCB DLBCL in patients treated with R-CHOP. BCL2 copy-number gain predicts inferior survival in the ABC subtype. Translocation of BCL6 is more frequent in ABC subtype DLBCL, and in some studies it has been associated with improved survival. c-myc translocations occur in about 8-14% of DLBCL, NOS cases and are associated with inferior survival. Most studies have confirmed that c-myc and BCL2 Double-hit lymphomas are much more common among GCB subtype and are associated with inferior survival. c-myc and BCL6 translocations are more common in the ABC subtype.

Aims & Objectives

Aims:

1. To study the proportion of cases of Double Expressors (DE) in Diffuse Large B-Cell Lymphomas, not otherwise specified (DLBCL, NOS) in a tertiary care setting.

Objectives:

1. 2. To determine the proportion of cases of Double Expressors which show co- expression of BCL2 and c-myc proteins in cases of DLBCL, NOS.
2. To characterize the patients according to their age-groups, gender, date of diagnosis and transformation from a lower grade lymphoma and to report these numbers.
3. To correlate, when possible, with the results of Fluorescent In-Situ Hybridization (FISH) to detect cases that harbor a c-myc translocation also showing a BCL2 translocation therefore belonging in the category of Double-Hit (DH) lymphoma.
4. To associate between the diagnosis of DE status and the clinical outcome of the patient at a minimum follow-up period of 1-year post diagnosis (regression) of primary tumor, appearance of new lesions and CNS metastases or death.

Table No. 39a: Comparitive Review of Previous Studies undertaken

AUTHORS	Smith SM et. al	Green TM et. al.	Li L et. al.	Aggarwal L et. al.	Roh J et. al.	Hashmi AA et. al.	Lores BF et. al.
TOTAL	--	193	212	69	181	109	102
DEL	20-44%	29%	36	17	82	35.8%	19
DHL	2-7%	15%	--	--	--	--	10
MALE	--	--	--	--	--	59	----
FEMALE	--	--	--	--	--	50	--
ABC PHENOTYPE	DEL are common	--	89% DEL	--	--	58	75%
GCB PHENOTYPE	DHL are common	--	--	--	--	51	25%

Table No. 39b: Comparitive Review of Previous Studies undertaken

AUTHORS	Nowakowski GS et. al.	Hassan Met. al.	Savage KJ et. al.	Mohammed AA et. al.	Pena C et. al.	Ma Z et.
TOTAL	100	88	376	90	53	98
DEL	--	19	127	27	9	34
DHL	57%	--	--	7	--	14
MALE	--	56	--	49	34	--
FEMALE	--	32	--	41	19	--
ABC PHENOTYPE	--	51	56%	--	11%	--
GCB PHENOTYPE	--	37	44%	--	89%	--

Materials & Methods

Study site & setting: Department of Pathology, Deenanath Mangeshkar Hospital and Research Centre.

Study Population: All histologically diagnosed cases of Diffuse Large B-

Cell Lymphoma (DLBCL) diagnosed at Deenanath Mangeshkar Hospital and Research Centre

Study Design: Retro-prospective observational study

Study Duration: June 2019 – June 2021

Inclusion criteria

1. Morphologically diagnosed cases of DLBCL, NOS in Deenanath Mangeshkar Hospital.
2. Types of specimens include biopsy specimens from nodal and extra nodal sites.
3. Morphologically diagnosed cases of DLBCL, NOS records of which of IHC and/or FISH analyses are available.
4. Cases diagnosed within the time frame of 1st January 2015 and 30th June 2020.

Exclusion criteria

1. Staging bone-marrow biopsy specimens yet to be evaluated for primary tumor status.
2. DLBCL, NOS cases which are morphologically diagnosed which have not undergone a detailed IHC work-up.
3. Cases of DLBCL, NOS in which we do not have access to the paraffin blocks.

Study Methodology

Specimens from nodal and extra-nodal sites diagnosed as Diffuse Large B-Cell Lymphoma, not otherwise specified (DLBCL, NOS) preserved as paraffin blocks were subjected to Immunohistochemical (IHC) testing for determination of c-myc and BCL2 protein expression. The data pertaining to the demographic profile of these patients was taken from the Histopathology registers and internal hospital software - AmritaHIS and MRD viewer.

In co-ordination with the clinical co-guide, the outcome of the patients at a minimum follow-up of 1-year post diagnosis will be documented in terms of clinical status and imaging.

H&E Staining

H&E staining usually means staining of nuclei by oxidized haematoxyline (haematin) through mordant (chelate) bonds of metals such as aluminium, followed by counterstaining by the Xanthene dye Eosin, which colours in various shades, different tissue fibres and cytoplasm.

IHC testing

Sample: IHC was done on 10% formalin fixed, paraffin embedded sections. (Biopsy tissue in 10% formalin/ paraffin blocks.) Optimal thickness of paraffin sections was approximately 3 to 4µ.

Antibodies used

Table No.2: List of antibodies used

Primary Antibodies	***Company***	***Clone***	***Positive Control***	***Interpretation***
BCL2	DAKOCYTOMATION	124	Tonsil	Membranous and/or cytoplasmic
BCL6 PG-Tonsil Nuclear	DAKOCYTOMATION	B6p	Tonsil	Nuclear
c-myc	PATHNSITU	EP121	Tonsil	Nuclear
CD10	DAKOCYTOMATION	56C6	Tonsil	Membranous
MUM1	DAKOCYTOMATION	MUM1p	Tonsil	Nuclear

Procedural steps

- The sections were deparaffinized by placing in oven at 60ºC (±2) for 5-10 minutes.
- The slides were kept in a Pascal jar which was filled with Target retrieval solution high pH or low pH. (pH-6.1, pH-9) The Pascal jar was then kept in Pascal system/ microwave and heated as follows: Pascal 25-35min (average 30 min) after adding 500ml D/W to Pascal. After the beep, the slides were allowed to cool at room temperature for 10 to 20 min.

- Slide cradle was washed in running tap water for 2 to 3min and then transferred to wash buffer immediately. The buffer wash was done in a moist chamber for 5 min. Peroxidase blocking solution was added for 5-7mins and buffer washed in moist chamber for 5- 10mins.
- Primary antibody (BCL2, BCL6 or c-myc) were added in dilution and incubated in the moist chamber for 1 hour. 3 changes Tris's buffer (buffered saline solution containing Tween20, pH 7.6±0.1) wash in moist chamber for 5 mins.
- Secondary antibody (Envisionflex) was added for 30 mins. 3 changes of Tris buffer wash in moist chamber for 5mins.
- DAB chromogen reagent (Diaminobenzidine tetrahydrochloride) was added and observed for 10mins for the colour to develop.
- Slides were washed in running water and counter stained with Harris's Hematoxyline, dehydrated, cleared and then mounted.

Reporting guidelines

BCL2 is considered positive if 50% of the tumour cells are positive, and c-myc is considered positive if 40% of the tumour cell nuclei are positive. Co-expression of these two proteins is seen in Double Expressers (WHO Classification of Tumors of Haematopoietic and Lymphoid Tissues, 2017 Revised Edition.)

Sample size with Justification

Formula used for sample size estimation:

To estimate proportion p in the study population, sample size required is $n=p(1-p)[(Z\alpha+Z1-\beta)/(p-p0)]2$ where

- n is sample size
- p0 is the comparison value =0.62 (62% incidence of DEL) &
- α is Type I error =5%; zα=1.96 two sided, Zα =1.64 one sided
- β is Type II error, 1−β is power; Z1−β=0.84 for 1−β=80%
- Effect size= (p-p0) = ±0.125(12.5%)

Based on the literature, assuming incidence of Double Expressor lymphomas (DEL) diagnosed by Immunohistochemistry, the subset of DLBCL, NOS and other High- Grade Lymphomas to be 62% with ± 12.5% allowable error, at confidence level 95% and power equal to 80%, the required sample size is 118 patients with DLBCL, NOS.

Statistical methods

Statistical analysis has been carried out with the help of SPSS (version 20) for Windows package (SPSS Science, Chicago, IL, USA). The description of the data will be done in form of arithmetic mean +/- SD for quantitative data while in the form of frequencies (%) for qualitative (categorical) data. P-values of <0.05 will be considered significant. For quantitative data, Unpaired Student's t-test will be used to test statistical significance of difference between means of variables among two independent groups. For comparison of categorical variables (i.e to examine the associations between qualitative/quantitative variables), chi-square test will be used if the number of elements in each cell are 5 or higher and Fisher's exact test, otherwise.

Observations & Results

The study performed was a Retro-prospective observational analysis.

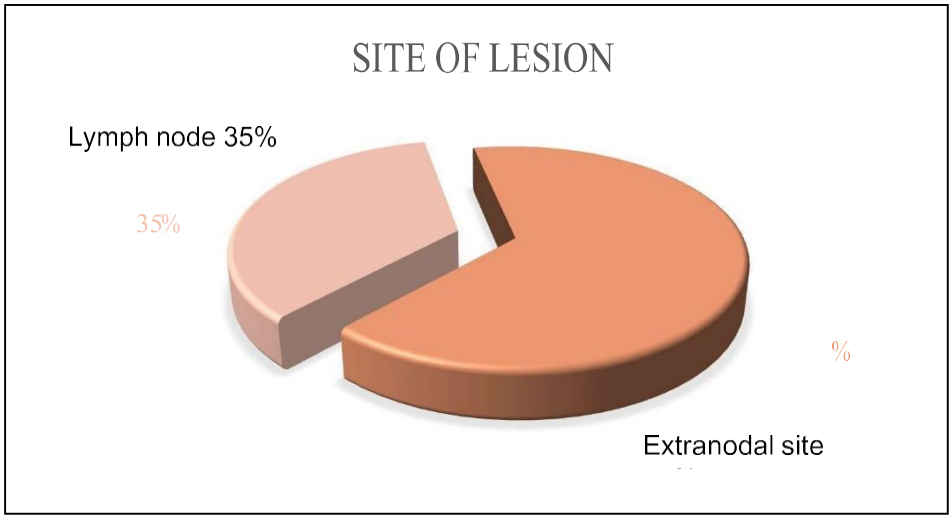
127 cases of DLBCL, NOS were diagnosed and evaluated at the Department of Pathology, Deenanath Mangeshkar Hospital and Research Centre.

Cases of DLBCL, NOS were collected within the time frame of 1st January, 2015 and 30th June, 2020.

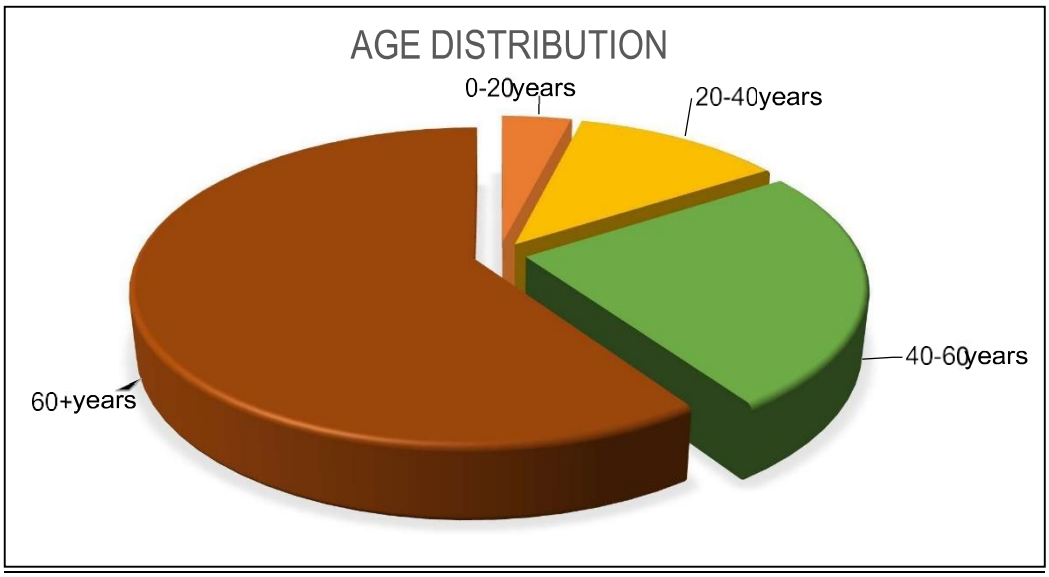
Observations

Table No.3: Sex-wise distribution of cases

Sex	Number	Percentage
Male	87	69%
Female	40	31%



Graph No. 1: Anatomical



Graph No.2: Age distribution of DLBCL, NOS cases

Table No. 4: Expression of IHC markers

IHC marker expression	Positive	Percentage	Negative	Percentage	Total
BCL2	110	88%	15	12%	125
c-myc	54	44.6%	66	55.3%	121
BCL6	74	73.2%	27	26.7%	101
CD10	50	43.8%	64	56.1%	114
MUM1	44	52.3%	40	47.6%	84

Double Expressors: Cases of DLBCL, NOS showing co-expression of BCL2 & c- myc, or BCL6 & c-myc are defined as DE whereas those cases showing co- expression of all 3 are called Triple Expressors (TEL.)

Table No. 5: Percentage of Double and Triple Expressors	
Expressors	Percentage
Double Expressors	66.929
Triple Expressors	25.197

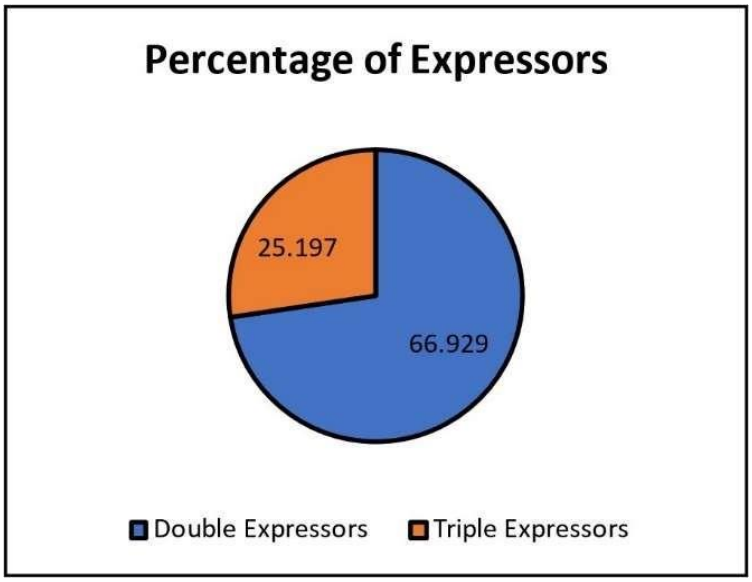
Deriving from the previously mentioned Aims and Objectives, results from the statistical analysis are listed as follows:

A total of 127 cases were studied. 125 cases were tested for BCL2, 121 cases were tested for c-myc and 101 cases were tested for BCL6 using Immunohistochemistry.

48/127 cases showed double expression of BCL2 and c-myc. 37/127 cases showed double expression of BCL6 and c-myc. Together, 85/125 cases were noted to be Double Expressors.

32/127 cases showed expression of all 3 markers and were noted to be Triple Expressors.

Table No. 6: Objective 1 To determine the proportion of cases of Double Expressors which show co-expression of BCL2 and c-myc proteins in cases of DLBCL, NOS.		
Expressors	Cases	Percentage
c-myc & BCL2	48	37.795
c-myc & BCL6	37	29.134
Total Double Expressors	85	66.929
c-myc, BCL2 & BCL6	32	25.197
Total No. of Cases N	127	



Graph No. 3: Percentage of Expressors

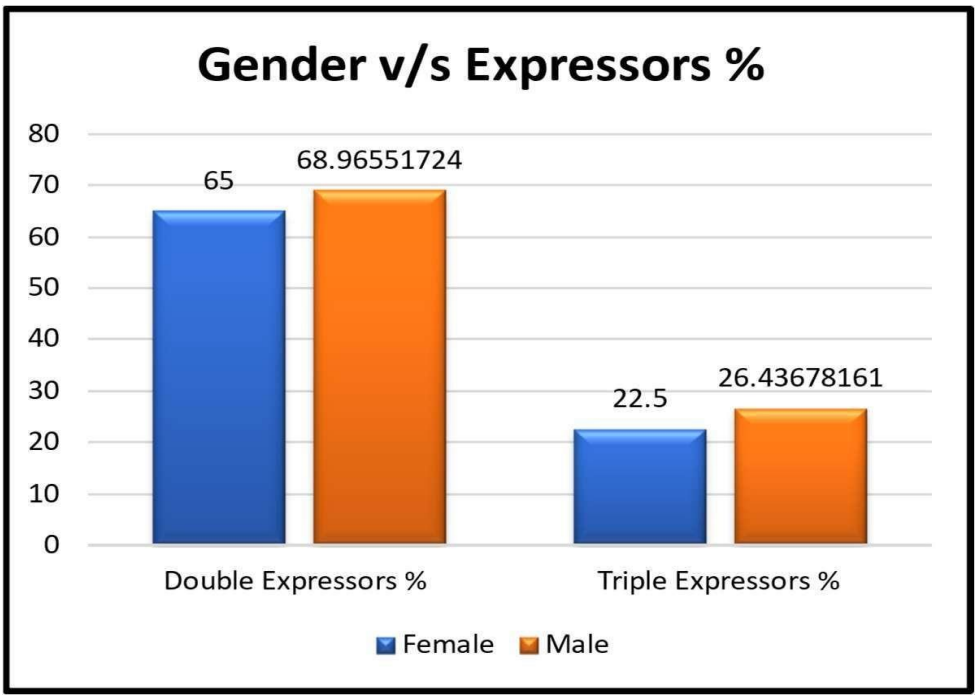
117 blocks were tested for c-myc, with BCL2 or/and BCL6 to determine Double and Triple Expressor status.

Out of a total of 85 Double Expressors, 60 cases were male and 26 cases were female; whereas out of 32 Triple Expressors, 23 cases were male and 9 cases were female.

Table No. 7: Objective 2. To characterize the patients according to their age-groups, gender, date of diagnosis and transformation from a lower grade lymphoma and to report these numbers.

Gender	Double Expressors	Triple Expressors	Total
Female	26	9	35
Male	60	23	83
Total	85	32	117

Chi Sq Value =	0.0496528
p Value =	0.8236686
df =	1



Graph No. 4: Percentage of Expressors Vs Gender

Chi Sq value is not significant. p-value is greater than 0.05. Hence the Null Hypothesis is retained. There is no significant association between Gender and (D or T) Levels of Expressors. Expressors are not associated with the Gender. Both the Genders are equally affected.

Table No. 8: Proportion of Expressors Vs Gender

Gender	Double Expressors %	Triple Expressors %	N
Female	65.000	22.500	40
Male	68.966	26.437	87
Total			127

Table no. 9: Proportion of Expressors Vs Gender

Gender	Double Expressors %	Triple Expressors %
Female	65.000	22.500
Male	68.966	26.437

Chi Sq value is not significant. p-value is greater than 0.05. Hence the Null Hypothesis is retained. There is no significant association between Age and (D or T) Levels of Expressors. Expressors are not associated with the Age. Both the Age groups are equally affected.

According to Age

Out of 85 cases of Double Expressors, 46 cases were of greater than 60 years of age 39 were less than 60 years of age. Out of 32 cases of Triple Expressors, 17 cases were over 60 years and 15 cases were less than 60 years of age.

Table No. 10: Proportion of Expressors Vs Age

Age	Double Expressors %	Triple Expressors %	N
More than 60	61.333	22.667	75
Less than or equal to 60	75.000	28.846	52
Total			127

Table No. 11: Proportion of Expressors Vs Age

Age	Double Expressors %	Triple Expressors %
More than 60	61.333	22.667
Less than or equal to 60	75.000	28.846

Table No. 12: Distribution of Expressors in Females

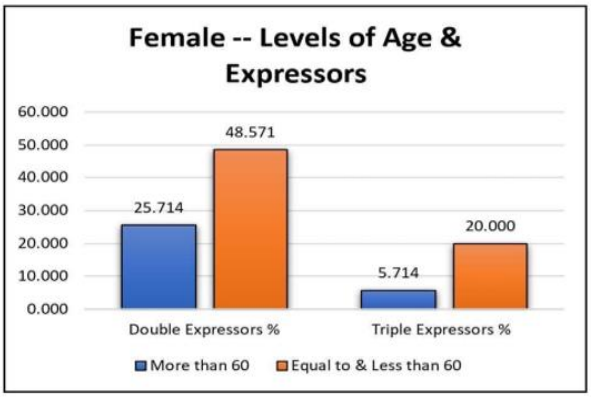
Female	Double Expressors	Triple Expressors	Tot al
More than 60	9	2	11
Equal to & less than 60	17	7	24
Total	26	9	35

Table No. 13: Distribution of Expressors in Males			
Male	Double Expressors	Triple Expressors	Total
More than 60	38	15	53
Equal to & less than 60	22	8	30
Total	60	23	83

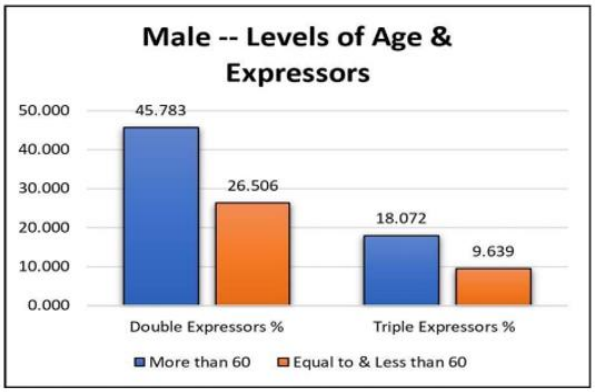
Table No. 14: Proportions of Expressors In Age Groups in Males		
Male	Double Expressors %	Triple Expressors %
More than 60	45.783	18.072
Equal to & less than 60	26.506	9.639

Table No. 15: Proportions of Expressors In Age Groups in Females		
Female	Double Expressors %	Triple Expressors %
More than 60	25.714	5.714
Equal to & less than 60	48.571	20.000

Graph No. 5: Proportion of Expressors in Age Groups in Females

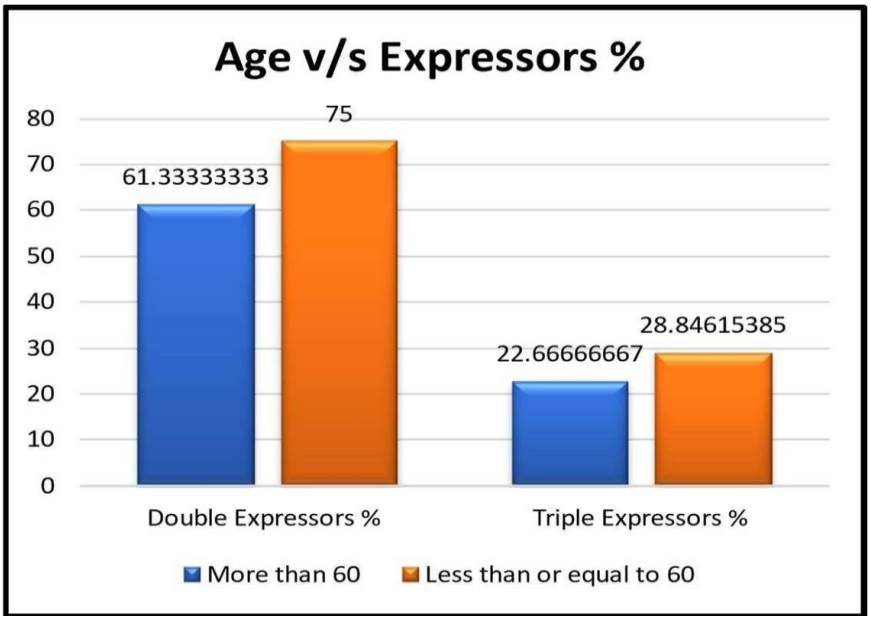


Graph No. 6: Proportion of Expressors in Age Groups in Males



Chi Sq Value =	0.4764795	Levels of age in Females & Type of Expressors are not associated
p Value =	0.4900213	
df =	1	

C Chi Sq Value =	0.0255711	Levels of age in Males & Type of Expressors are not associated.
p Value =	0.8729524	
df =	1	



Graph No. 7: Proportion of Expressors Vs Age

According to Cell of Origin

100 cases out of 127 cases were evaluated for Cell of Origin using the Hans algorithm. Few of the cases were not evaluated due to inadequate residual tissue in the block, so the focus was placed on expression of c-myc and BCL2. 66 cases out of 100 were of the GCB phenotype (66%) and 34 cases were of the ABC phenotype (34%.)

Table No. 16: Double & Triple Expressors in ABC Phenotype

ABC -- Proportion of cases of Double & Triple Expressors

c-myc & BCL2	c-myc & BCL6	Total Double Expressors	c-myc, BCL2 & BCL6	Total No. of Cases N
15	8	23	8	31

Table No. 17: Percentage of Expressors in ABC Phenotype

Expressors	Percentage
Double Expressors	67.647
Triple Expressors	23.529

Table No. 18: Percentage of Expressors in ABC Phenotype

Expressors	ABC Cases	Percentage
c-myc & BCL2	15	44.118
c-myc & BCL6	8	23.529
Total Double Expressors	23	67.647
c-myc, BCL2 & BCL6	8	23.529
Total No. of Cases N	34	

Graph No. 8: Percentage of Expressors in ABC Phenotype

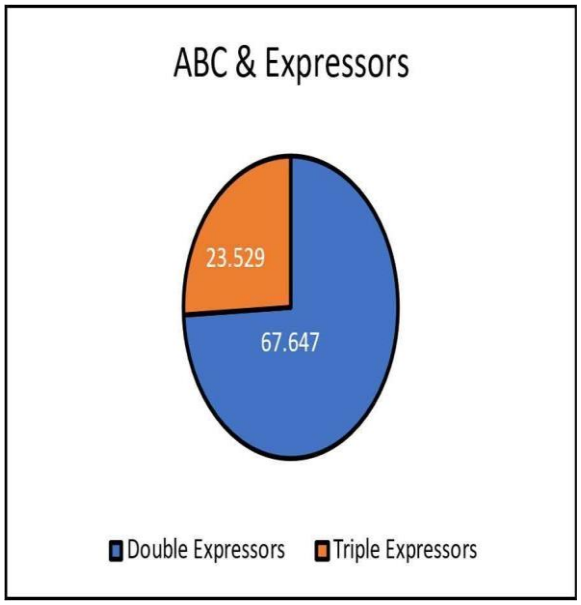


Table No. 19: Percentage of Expressors in GCB Phenotype

Expressors	GCB Cases	Percentage
c-myc & BCL2	27	40.909
c-myc & BCL6	25	37.879
Total Double Expressors	52	78.788
c-myc, BCL2 & BCL6	21	31.818
Total No. of Cases N	66	

Table No. 20 : Double and Triple Expressors in GCB Phenotype

GCB -- Proportion of cases of Double & Triple Expressors				
c-myc & BCL2	c-myc & BCL6	Total Double Expressors	c-myc, BCL2 & BCL6	Total No. of Cases N
27	25	52	21	73

Graph No. 9: Percentage of Expressors in GCB Phenotype

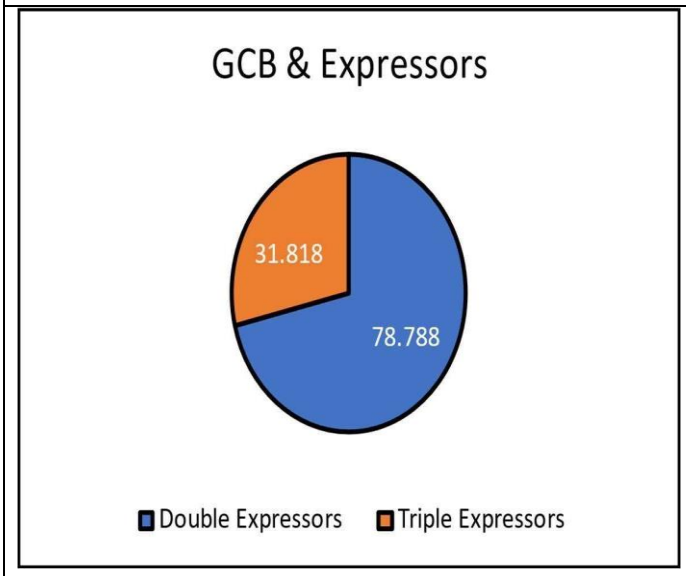


Table No. 21: Percentage of Expressors in GCB Phenotype

Expressors	Percentage
Double Expressors	78.788
Triple Expressors	31.818

Table No. 22: Objective 3. To correlate, when possible, with the results of Fluorescent In-Situ Hybridization (FISH) to detect cases that harbor a c-myc translocation also showing a BCL2 translocation therefore belonging in the category of Double-Hit (DH) lymphoma

	BCL2	c-myc	FISH TRANSLOCATION
	POSITIVE	20-30%	NO
	POSITIVE	60%	NO
	POSITIVE	60%	NO
	POSITIVE	20-30%	NO
	POSITIVE	20%	NO
	POSITIVE	20-25%	NO
	POSITIVE	30-35%	NO
	POSITIVE	35-40%	NO
	POSITIVE	40-50%	NO
	POSITIVE	30%	NO
	POSITIVE	40-50%	NO
	POSITIVE	30-36%	NO
	POSITIVE	30-40%	NO
	Focal	70%	NO
	POSITIVE	45-50%	POSITIVE for c-myc gene rearrangement.
	POSITIVE	80%	POSITIVE for c-myc gene rearrangement.

Table No. 23: Cases Positive for translocation by FISH

	BCL2 +ve	c-myc +ve
FISH +ve	2	2
FISH ve	15	7

The Fisher exact test statistic value is 0.5906. The result is not significant at $p < .05$

No association between positive FISH test and detection that harbor a c-myc translocation also showing a BCL2 translocation therefore belonging in the category of Double-Hit (DH) lymphoma.

Transformation from Low grade non-Hodgkin's lymphoma

8 out of 127 cases were observed to be transformations from low grade non- Hodgkin's lymphoma. 4 cases had been earlier diagnosed with Follicular lymphoma, 1 out of 8 cases evolved from a Splenic Marginal Zone lymphoma and 1 case evolved from a Mantle Cell lymphoma. 2 out of 8 cases transformed from unspecified low-grade NHLs.

Post 1 year of diagnosis

All cases were followed up for up to 1-year post diagnosis. 48 cases were evaluated with the help of Radiological Imaging vis-à-vis. CT, PET-CT, Ultrasound etc. Graph No.12 shows charting of the response to chemotherapy assessed by imaging at the time of last follow up within 1 year of diagnosis.

21 cases were still on therapeutic chemotherapy at the time of the last visit within 1-year post diagnosis. None of these patients presented with any new complaints at the time of visit. It was assumed that the patients were discharged in a stable condition.

13 patients were on palliative chemotherapy at the time of the last visit during the 1 year follow up period. Out of these, 1 patient presented with a persistent fungating mass, and 1 patient presented with persistently enlarged cervical lymph nodes, both suggestive of poor response to chemotherapy.

33 patients did not receive chemotherapy. These could not be followed up with, up to 1-year post chemotherapy. Some cases were referred to a secondary centre or Hematologist outside of Deenanath Mangeshkar Hospital. Few of the cases were discharged post diagnosis on account of aggressive nature of disease with comfort care.

12 patients died within 1-year post diagnosis. 4 out of these 12 were on palliation.

4 cases were receiving therapeutic chemotherapy at the time of death. Death occurred due to Neutropenic sepsis or lower respiratory tract infection. 4 patients died without commencing chemotherapy.

Table No. 24: Cell of Origin & Prognosis

CELL OF ORIGIN	Poor Prognosis	Good Prognosis	Total
ABC subtype	4	5	9
GCB subtype	5	10	15

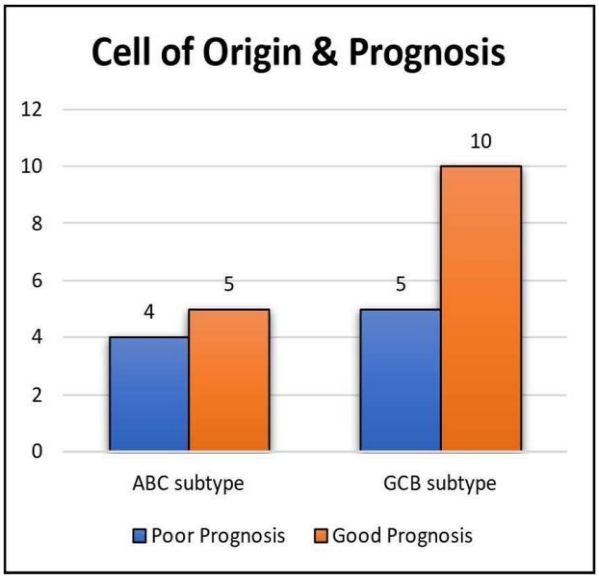
Table No. 25: Percentage of Cell of Origin & Prognosis

CELL OF ORIGIN	Poor Prognosis %	Good Prognosis %
ABC subtype	44.44	55.56
GCB subtype	33.33	66.67

CELL OF ORIGIN	Poor Prognosis	Percentage	Good Prognosis	Percentage	Total
ABC subtype	4	44.444	5	55.556	9
GCB subtype	5	33.333	10	66.667	15

Table No. 26: Percentage of Cell of Origin & Prognosis

Graph No. 10: Cell of Origin & Prognosis



Graph No. 11: Percentage of Cell of Origin & Prognosis

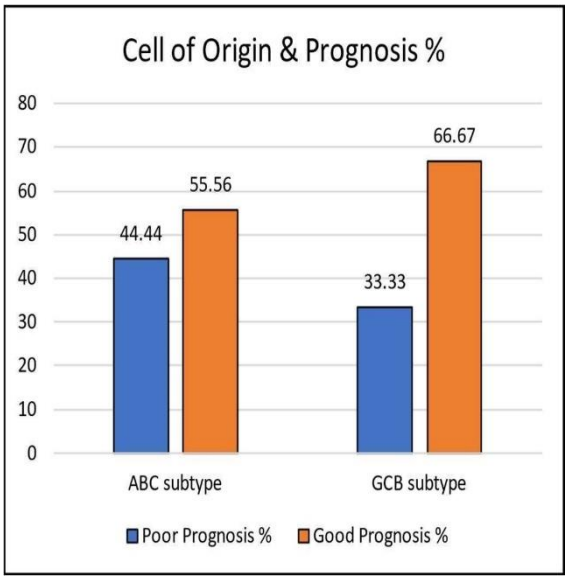


Table No. 27: Outcome of cases 1 year post diagnosis based on imaging	
Clinical Status after 1 Year	Number
Regression of Lesion	19
Complete Metabolic Response	7
Poor Response	7
No Residual Lesion	6
Appearance of New Lesion	3
Regression of Lesion and appearance of New Lesion	2
Relapse Lesion	2
Partial Metabolic Response	1
Complete Remission	1

4. To associate between the diagnosis of DE status and the clinical outcome of the patient at a minimum follow up period of 1-year post diagnosis (regression) of primary tumor, appearance of new lesions and CNS metastases or death.)

Graph No. 12: Outcome of cases 1 year post diagnosis based on imaging

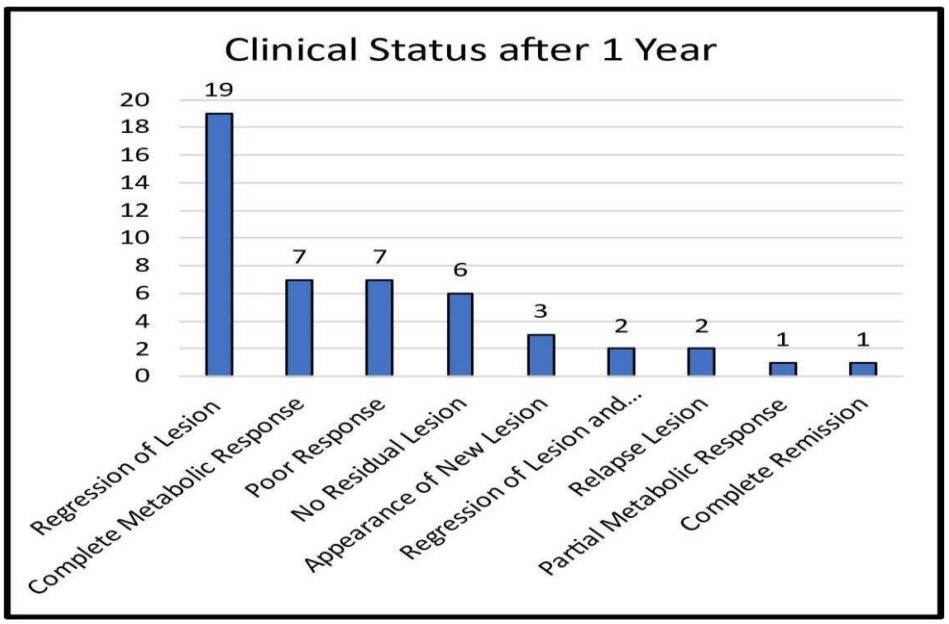


Table No. 28: Prognosis Vs. Tumor site

Specimen / Prognosis	Bone & Muscle	CNS	Endocrine	GI	Head & Neck	Lymph Node	Skin	Total
Good Prognosis	10	2	1	13	10	14	1	51
Poor Prognosis	2	2	0	2	1	3	0	10
Dropped out of Prognosis	1	0	0	3	1	3	0	8
Lost to Follow Up	7	2	1	12	4	8	0	34
No Prognosis	3	1	0	8	1	10	1	24

Table No. 29: Prognosis Vs. Tumor site % Based on Total Cases (127)

Specimen / Prognosis	Bone & Muscle	CNS	Endocrine	GI	Head & Neck	Lymph Node	Skin
Good Prognosis	7.874	1.575	0.787	10.236	7.874	11.024	0.787
Poor Prognosis	1.575	1.575	0.000	1.575	0.787	2.362	0.000
Dropped out of Prognosis	0.787	0.000	0.000	2.362	0.787	2.362	0.000
Lost to Follow Up	5.512	1.575	0.787	9.449	3.150	6.299	0.000
No Prognosis	2.362	0.787	0.000	6.299	0.787	7.874	0.787

Table No. 31: Intensity of c-myc Vs. Prognosis % Based on Total Cases (127)

c-myc / Prognosis	Borderline	Low	Negative	Positive	Strong Positive
Good Prognosis	6.299	12.598	4.724	11.811	2.362
Poor Prognosis	3.150	4.724	0.787	7.087	1.575
Dropped out of Prognosis	1.575	2.362	0.000	2.362	0.000
Lost to Follow Up	4.724	5.512	3.150	10.236	2.362
No Prognosis	0.000	3.150	1.575	3.150	0.000

Table No. 32: Prognosis Vs. Cell of Origin phenotype					
Cell of Origin / Prognosis	ABC Type	GCB Type	Total (Within Group)	Total (All Cases 127) ABC & GCB	
Good Prognosis	10	28	38	7.874	22.047
Poor Prognosis	12	15	27	9.449	11.811
Dropped out of Prognosis	4	4	8	3.150	3.150
Lost to Follow Up	8	18	26	6.299	14.173
No Prognosis	0	1	1	0.000	0.787

Table No. 33: Prognosis Vs. Cell of Origin phenotype % based on Total within group		
Cell of Origin / Prognosis	ABC Type	GCB Type
Good Prognosis	26.315789	73.684211
Poor Prognosis	44.444444	55.555556
Dropped out of Prognosis	50	50
Lost to Follow Up	30.769231	69.230769
No Prognosis	0	100

	Table No. 34: Cases showing Poor Prognosis on the basis of Specimen, c- myc & Cell of Origin							
	ABC				GCB			
c- myc	Low	Borderline	Positive	Strong Positive	Low	Borderline	Positive	Strong Positive
	2	3	4	0	2	1	4	2

Table No. 35: Cases showing Good Prognosis on the basis of Specimen, c- myc & Cell of Origin				
	ABC		GCB	
c- myc	Positive	Strong Positive	Positive	Strong Positive
	6	0	7	2

Discussion

A total of 127 cases were studied. 125 cases were tested for BCL2, 121 cases were tested for cmyc and 101 cases were tested for BCL6 using Immunohistochemistry.

Age-Sex distribution

Out of the total 127 cases of DLBCL, 87 cases making up 69% cases were male, and 40 cases making up 31% of the cases were female.

Thus, a male predominance was noted in the total number of cases of DLBCL, NOS studied.

The cases of DLBCL were classified according to their age. 4 age groups were defined. A maximum of 59% of cases were seen in patients above 60 years of age, and this group represented the maximum number of cases out of all age groups. In descending order, the cases were distributed as follows: 24.3% (40- 60 years), 11.8% (20-40 years) and 3.9% (Below 20 years of age.) In sync with data retrieved from all over the world, the cases in our setting also fell into similar age groups. The data was representative of the fact that DLBCL, NOS is a disease of older age groups.

One striking finding was a case of a Temporal lobe lesion in a 6-year-old male child, consistent with DLBCL, NOS.

Specimens: Samples were collected in the form of Trucut biopsies, excisional biopsies, excision of lesion, bowel resection specimens, neurosurgery tumors etc. Sites were broadly divided into nodal and extranodal sites. 65% of the specimens belonged to extranodal locations, whereas 35% were lymph nodal tissue from multiple sites. Lesions from the CNS and tonsils were considered as extranodal sites as well.

32 cases were lymph node specimens in the form of excision and incision biopsy specimens. 52 cases were linear-core biopsies from both nodal and extra-nodal sites. 6 cases were excisional and stereotactic biopsies from CNS lesions.

14 cases were from Gastrointestinal lesions, and of these, most common were from gastric ulcers. 19 cases were head and neck lesions, including tonsillar masses, base of tongue soft tissue lesions, thyroid lesions, and nasopharyngeal masses. A single lesion each was seen as a wrist soft tissue lesion and testicular tumor respectively.

Table No. 36: Comparison between DEL case numbers	Verma A, Mehta A et. al.			Present study	
	DLBCL	DHL	DEL	DLBCL	DEL
Total	145	7	20	127	85
Male	86 (59.3%)	1 (14.3%)	12 (60%)	87	60 (68.9%)
Female	59 (40.7%)	6 (85.7%)	8 (40%)	40	26 (65%)

Of all Gastrointestinal tumors, a majority was seen in the stomach. Similarly, a large number of tumors were localized to the head-neck region.

Being a tertiary care centre, maximum cases were diagnosed on linear core biopsies. It may be assumed that if most of the tissues extracted were adequate and representative of the larger lesion in form of excision specimens, Immunohistochemical staining may be optimal and thus provide a more accurate result.

Deriving from the previously mentioned Aims and Objectives, results from the statistical analysis are listed as follows:

48/127 cases showed double expression of BCL2, and c-myc. 37/127 cases showed double expression of BCL6 and c-myc. These represented 37.7% and 29.1% of the total cases respectively. Together, 85/125 cases were noted to be Double Expressors and represented 66.9% of the total cases. Thus, we noted a greater proportion of Double Expressors of BCL2 and c-myc as compared to BCL6 and c-myc. This was comparable to the research by Petrich AM et al.

32/127 cases showed expression of all 3 markers and were noted to be Triple Expressors. They represented 25.1% of the total cases of DLBCL, NOS.

Out of a total of 85 Double Expressors, 60 cases were male, and 26 cases were female; whereas out of 32 Triple Expressors, 23 cases were male and 9 cases were female. The proportion of Double Expressors in males was therefore calculated as 68.9% and that in females was calculated as 65%. The proportion of Triple Expressors in males was calculated as 26.4% and that in females was calculated as 22.5%. It was thus observed from this study that males showed a greater proportion of Double Expressors and Triple Expressors. If these cases were hypothetically evaluated by FISH, they may be used to challenge the study by Verma A et. al. who reported higher incidence of Double Hit Lymphomas in females.

When classified by age and sex, it was noticed that 48.57% of females less than 60 years and 45.78% males over 60 showed higher proportion of Double Expressors. Whereas, 26.5% males less than 60 years and 25.7% females over 60 years showed a higher proportion of Triple Expressors.

According to Age

Out of 85 cases of Double Expressors, 46 cases were of greater than 60 years of age 39 were less than 60 years of age. Out of 32 cases of Triple Expressors, 17 cases were over 60 years, and 15 cases were less than 60 years of age.

When proportion of the Double Expressors was calculated according to their age groups, 61.33% were over 60 years and 75% were less than 60 years of age.

When proportion of the Triple Expressors was calculated according to their age groups, 22.66% were over 60 years and 28.8% were less than 60 years of age. Thus, concluded from this study, Double Expressors and Triple expressors occurred more in patients below 60 years of age.

According to Cell of Origin

100 cases out of 127 cases were evaluated for Cell of Origin using the Hans algorithm. Few of the cases were not evaluated due to funding limitations so the focus was placed on expression of cmyc and BCL2. 66 cases out of 100 were of the GCB phenotype (66%) and 34 cases were of the ABC phenotype (34%.) Our findings corroborate literature published by the WHO and Western World literature reporting higher incidence of GCB phenotype than ABC phenotype among DLBCL, NOS cases. However, the study was not in agreement with the findings of studies by Hassan M, et. al. and Hashmi AA, et. al. who reported higher incidence of ABC phenotype in the Asian countries.

52 Double Expressors and 21 Triple Expressors were of the GCB phenotype. Therefore, 78.78% were GCB Double Expressor lymphomas and 31.81% were GCB Triple Expressor lymphomas. 23 Double expressors and 8 Triple Expressors were of the ABC phenotype. Therefore 67.64% were ABC Double Expressor Lymphomas and 23.52% were ABC Triple Expressor lymphomas.

These results were in contrast to the studies referenced by the WHO, Smith SM et. al, Li L et. al and Lores BF et. al.

Table 37: Comparison between DEL in Cell of Origin subtype

	Smith SM et. al.	Li L, Zhang X	Lores BF	Present study
Total DEL	100	36	100	85
GCB subtype	37%	11%	25%	52 (61%)
ABC subtype	63%	89%	75%	23 (27%)

The WHO guidelines suggest that 18-43% of the DLBCL cases are reported to be DE lymphomas, and, 20% of DE lymphomas are reported to be DH lymphomas.

Out of 127 cases of DLBCL, 16 cases underwent testing by FISH. 7/16 cases were Double Expressors for BCL2 and c-myc by IHC. 2/7 (28.5%) cases were positive for a genetic aberration of c-myc gene, and were thus considered to be Double Hit Lymphomas, this implying that in this study 28.5% of DELs were Double Hit lymphomas. To estimate the percentage of Double Hit out of the total of 127 cases studied, containing 85 DE cases, would imply that 28.5% of 85 cases, 24 cases could be expected to be Double Hit Lymphomas provided the other variables remain constant and comparable. Hence, 19% of the total cases of DLBCL, NOS was the estimated population proportion of Double Hit Lymphomas. Therefore, the outcome of our study is comparable to the data published by the WHO.

Table 38: Comparison between DEL and DHL proportions

	WHO data	Smith SM	Green TM et.al.	Pena et. al.	Mohammed	Savage KJ et. al.	Ma Z et. al	Aggarwal et. al	Li L et. al.	Present Study
DEL	18-43%	20-30%	29%	7.5%	(30%)	(30%)	34.7%	44.7%	41%	66.9%
DHL	4-7%	5-12%	6%	--	--	--	14.29%	--	--	Approx. 19%

Out of 2 cases of DHL, 1 case was of the ABC phenotype. It was impossible to determine Cell of Origin of the second case as the paraffin block was unavailable for BCL6 IHC testing.

Transformation from Low grade non-Hodgkin's lymphoma

8 out of 127 cases (6.2%) were observed to be transformations from low grade non-Hodgkin's lymphoma. 4 (3.1%) cases had been earlier diagnosed with Follicular lymphoma, 1 out of 8 cases evolved from a Splenic Marginal Zone lymphoma and 1 (0.78%) case evolved from a Mantle Cell lymphoma. 2 (1.5%) out of 8 cases transformed from unspecified low-grade NHLs. Hence our data shows a trend of Follicular lymphomas transforming into higher grade NHLs.

Post 1 year of diagnosis

All cases were followed up for upto 1-year post diagnosis. 48 cases were evaluated with the help of Radiological Imaging vis-à-vis. CT, PET-CT, Ultrasound etc. Graph No.12 shows charting of the response to chemotherapy assessed by imaging at the time of last follow up within 1 year of diagnosis.

21 cases were still on therapeutic chemotherapy at the time of the last visit within 1-year post diagnosis. None of these patients presented with any new complaints at the time of visit. It was assumed that the patients were discharged in a stable condition and had a good prognosis.

13 patients were on palliative chemotherapy at the time of the last visit during the 1 year follow up period. Out of these, 1 patient presented with a persistent fungating mass, and 1 patient presented with persistently enlarged cervical lymph nodes, both suggestive of poor response to chemotherapy and therefore poor prognosis.

33 patients did not receive chemotherapy. These could not be followed up with, upto 1-year post chemotherapy. Some cases were referred to a secondary centre or Hematologist outside of Deenanath Mangeshkar Hospital. Few of the cases were discharged post diagnosis on account of aggressive nature of disease with comfort care.

12 patients died within 1-year post diagnosis. 4 out of these 12 were on palliation. 4 cases were receiving therapeutic chemotherapy at the time of death. Death occurred due to Neutropenic sepsis or lower respiratory tract infection. 4 patients died without commencing chemotherapy. From the results of the study, we attempted to make an association between the prognosis of the patient as evaluated at 1-year post diagnosis and the Cell of Origin.

66.67% of the GCB phenotype cases and 55.56% of the ABC phenotype cases showed good prognosis post chemotherapy. Therefore DLBCL, NOS cases of the GCB phenotype showed a better response to chemotherapy and had a better prognosis, as compared to their ABC phenotype counterparts.

The study by Kluk MJ et al. focused on the importance of developing predictive biomarkers in DLBCLs where knowledge of a biomarker impacts an initial or subsequent therapeutic decision. They identified groups of patients with inferior clinical outcome when treated with R-CHOP and observed that c-myc protein expression had a correlation with transcription and prognostic outcome. Developing on this, we analyzed patients according to the percentage of cells staining for c-myc by Immunohistochemistry. We divided the cases into 4 categories: Positive (40-70%), Strong Positive (70%+), Borderline (30-40%) and Low (Less than 30%) in order to compare their outcome at 1-year post diagnosis.

Low c-myc: A maximum of 12.5% of all cases showed good prognosis. 4.7% showed poor prognosis. 40% cases showed complete or near-complete metabolic response, or remission when evaluated. Majority of these cases showed GCB phenotype. 2 (4%) cases showed partial metabolic response. 5 (10%) cases showed appearance of new lesions, suggestive of relapse. The cases were equally distributed among GCB and ABC phenotypes. The remaining cases could not be assessed as chemotherapy was on-going, or patients were lost to follow-up.

Borderline c-myc: 6.2% of all DLBCL, NOS cases showed a good response to treatment with no new complaints. Most of these cases had GCB phenotype. 3.1% cases showed a poor response to chemo with residual lesions. 1 case showed imaging suggestive of CNS relapse (Parietal lobe lesion) and showed GCB phenotype. 30% cells stained positive in this case. 1 case with 30-40% c- myc staining showed diffuse splenomegaly and had ABC phenotype. 1 patient showed diffuse skin toxicity after chemotherapy and had ABC phenotype. Data from the remaining cases was not retrieved, or patients were put on palliation, then lost to follow-up.

Positive c-myc: 46 cases fell into this category. 40 of these cases were Double Expressors. 15 (32.6%) cases showed complete regression of lesions and complete metabolic response post chemotherapy. Majority of these lesions had ABC phenotype. 7 (15.2%) cases showed signs of relapse, and these were equally both GCB and ABC phenotype. There was death of 5 patients and most of them showed

GCB phenotype. The highest, 7% of total cases showing poor prognosis belonged in this category. However, this group also showed maximum 11.8% cases showing good prognosis and response to chemotherapy. Data from the remaining cases was not retrieved, or patients were put on palliation, then lost to follow-up.

Strong positive c-myc: 8 cases fell into this category. 3 (37.5%) cases showed complete metabolic response and belonged to GCB phenotype. 2 (25%) cases showed relapse of disease and both belonged to GCB phenotype. Of these, 1 case with 80% c-myc staining showed CNS relapse. The remaining cases were lost to follow up.

Double-Hit lymphoma: Both cases diagnosed as DHL with c-myc rearrangement showed a complete metabolic response after chemotherapy. One case showed 40-45% staining with c-myc and the other case showed 80% staining for c-myc. The first case belonged to ABC phenotype. It was impossible to assess Cell of Origin for the second case due to lack of BCL6 staining. Concluding from Table 34, an equal number of 4 cases belonging to both GCB and ABC phenotypes, stained for c-myc between 40-70% by IHC and showed poor prognosis, however, 3 cases of ABC phenotype stained between 30-40% (borderline) and showed poor prognosis, as opposed to one case of GCB phenotype. 2 cases of GCB phenotype stained > 70% and showed poor prognosis as compared to none from ABC phenotype.

Table No. 35 showed 7 cases belonging to GCB phenotype staining 40-70% and 2 cases staining > 70% for c-myc showing good prognosis, whereas only 6 cases of ABC phenotype staining 40-70% showed good prognosis.

Thus concluding, both GCB and ABC phenotype showed equal cases with poor prognosis when c-myc stains between 40-70%, however, staining more than 70% in the GCB phenotype shows poor prognosis. Conversely, more cases of GCB phenotype staining between 40-70% showed good prognosis. Therefore, within the GCB phenotype, cases staining between 40–70% have better prognosis, that those staining more than 70%.

From these findings, it can be concluded that there is a strong correlation between the intensity of c-myc staining and rate of relapse. The lowest relapse rate of 10% was noted in the group with low c-myc staining followed by 15.2% in the group showing intensity between 40-70% and then 17.6% in the borderline group. The highest rate of relapse of 25% was seen in the group showing strong >70% staining for c-myc. Considering the rate of relapse being significantly high in the borderline group

(17.6%), there is a strong possibility these cases may also harbor a translocation of c-myc which might be missed if protein overexpression as observed by IHC does not meet the WHO reporting guideline of 40% to qualify for FISH testing. Therefore, our study indicates that cases showing borderline c-myc staining by IHC, especially of ABC phenotype, should be considered as potential candidates for Fluorescent in-Situ Hybridization for genetic aberration testing.

Summary and Conclusions

- The study was a retro-prospective observational study of 127 cases of Diffuse Large B-cell Lymphoma, Not otherwise specified (DLBCL, NOS.)
- 87 cases making up 69% cases were male, and 40 cases making up 31% of the cases were female. A male predominance was noted in the total number of cases of DLBCL, NOS studied.
- A maximum of 59% of cases were seen in patients above 60 years of age, and this group represented the maximum number of cases out of all age groups. The data was representative of the fact that DLBCL, NOS is a disease of older age groups.
- 65% of the cases were extra-nodal tumors, whereas 35% cases occurred in lymph nodes.
- 85/127 cases were noted to be Double Expressors and represented 66.9% of the total cases and 32/127 were noted to be Triple Expressors represented 25.1% of the total. Double Expressors of BCL2 and c-myc (48) were greater in number as compared to BCL6 and cmyc (37.)
- Males showed a greater proportion of Double Expressors - 68.9% and Triple Expressors - 26.4%, than their female counterparts.
- A unique trend - 48.57% of females less than 60 years and 45.78% males over 60 showing higher proportion of Double Expressors, 26.5% males less than 60 years and 25.7% females over 60 years showing a higher proportion of Triple Expressors, was noted.

- DE: 61.33% were over 60 years and 75% were less than 60 years of age.
    - TE: 22.66% were over 60 years and 28.8% were less than 60 years of age.
    - Thus, Double Expressors and Triple expressors were more frequently seen in patients below 60 years of age.
- Using the Hans Algorithm, a higher number of 66 cases were of the GCB phenotype (66%) and 34 cases were of the ABC phenotype (34%.)
- Within the GCB phenotype, 78.78% were Double Expressor lymphomas and 31.81% were Triple Expressor lymphomas. Within the ABC phenotype 67.64% were Double Expressor

Lymphomas and 23.52% Triple Expressor lymphomas. In our study, Double Expressors and Triple Expressors were seen more commonly in the cases with GCB phenotype.

- 4 out of 8 cases (50%) of transformations from low grade non-Hodgkin's lymphoma were earlier diagnosed with Follicular lymphoma. This was a trend noted.
- Out of 127 cases of DLBCL, 16 cases underwent testing by FISH. 7/16 (43.7%) cases were Double Expressors for BCL2 and c-myc by IHC. 2/7 (28.5%) cases were positive for a genetic aberration of c-myc gene, and were thus considered to be Double Hit Lymphomas. In our study, 19% of the total cases of DLBCL, NOS would be a crude estimate of the population proportion of Double Hit Lymphomas.
- The clinical status of patients at 1-year post diagnosis revealed that DLBCL, NOS cases of the GCB phenotype showed a better response to chemotherapy and had a better prognosis, as compared to their ABC phenotype counterparts.
- The highest 7% of total cases showing poor prognosis belonged in the cases staining between 40-70% for c-myc. This group also showed maximum 11.8% cases showing good prognosis and response to chemotherapy.
- More cases staining borderline positive for c-myc by IHC belonging to ABC phenotype showed poor prognosis.
- Post chemotherapy, the highest rate of relapse of 25% was seen in the group showing strong >70% staining for c-myc. There is a strong correlation between the intensity of c-myc staining and rate of relapse.
- Cases showing borderline positive c-myc staining by IHC belonging to either phenotype, should also be considered as potential candidates for Fluorescent in-Situ Hybridization for genetic aberration testing as the rate of relapse was 17.6% and significantly close to the relapse rate of those showing strong positivity.

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