# THE IMMUNOHISTOCHEMICAL STUDIES OF DOUBLE OR TRIPLE PROTEIN LYMPHOMA IN DIFFUSE LARGE B-CELL LYMPHOMA AND THEIR CORRELATION WITH CLINICOPATHOLOGICAL PARAMETERS

DR WAN NOR NAJMIYAH BT WAN ABDUL WAHAB

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PATHOLOGY (ANATOMIC PATHOLOGY)


SCHOOL OF MEDICAL SCIENCES

UNIVERSITI SAINS MALAYSIA

2017

# AKNOWLEDGEMENT

First and foremost, deepest thank you to Allah The Most Merciful and Most Gracious for all His blessings that make it possible for me to finish this dessertation.

My biggest gratitude goes to my supervisor, Dr Faezahtul Arbaeyah Hussain for all her guidance and efforts in helping me with this thesis. Thank you to my co-supervisor AP Dr Azlan for his support and encouragement.

Special thanks to the lecturers, colleagues and staffs of Pathology Department, especially Encik Rosli Jusoh and Puan Jamaliah who has helped me with the laboratory works. The expertise and knowledge shared very much appreciated.

Special dedications goes to my beloved husband, Encik Roslan Said and my daughter, Raudhatul Jannah for their understanding and endless support. My appreciation goes to my parent, parents in law and family members for their continous support and prayers.

This study was funded by Short Term Grant (STG 304/PPSP/613113038) from Pusat Pengajian Sains Perubatan, Universiti Sains Malaysia.

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABC | activated B-cell |
| COO | cell of origin |
| DLBCL | Diffuse Large B-cell Lymphoma |
| DH | Double Hit |
| DP | double protein lymphoma |
| ECOG | Eastern Cooperative Oncology Group |
| FISH | fluorescent in situ hybridization |
| GEP | gene expression profile |
| GCB | germinal center B-cell |
| IHC | immunohistochemistry |
| IPI | International Prognostic Index |
| LDH | Lactic dehydrogenase |
| NHL | Non-Hodgkin's Lymphoma |
| OS | overall survival |
| PFS | progression-free survival |
| PET | 18Fluorodeoxyglucose Positron Emission Tomography |

| | |
|---|---|
| WHO | World Health Organization |

# ABSTRAK

**Pengenalan dan objektif:** Dalam tahun-tahun kebelakangan ini, "double hit" dan "double protein" melibatkan penyusunan semula gen dan ekspresi protein MYC dan BCL2 dan/atau BCL6 adalah ungkapan yang paling banyak digunakan untuk menggambarkan faktor prognostik di dalam "diffuse large B-cell lymphoma (DLBCL)". Kajian ini adalah untuk menentukan kekerapan ekspresi 'double/triple protein lymphoma" dengan menggunakan immunohistokimia dan mengaitkan keputusannya dengan ciri-ciri klinikopatologikal dan klasifikasi sel asal.

**Bahan dan kaedah:** Kami telah menjalankan satu kajian keratan rentas dengan menggunakan 29 blok tisu parafin kes DLBCL. Semua sampel telah dinilai untuk ekspresi protein-protein MYC, BCL2 dan BCL6 oleh immunohistokimia. Di samping itu, subkumpulan daripada COO DLBCL telah ditentukan oleh ekspresi CD10, BCL6 dan MUM1 berdasarkan klasifikasi Hans.

**Keputusan**: Antara 29 kes, MYC, BCL2 dan BCL6 protein masing-masing telah dikesan dalam 72.4%, 62.1% dan 62.1% daripada pesakit. Ekspresi serentak (Positif MYC/ positif BCL2 dan/atau positif BCL6) dikesan di dalam 58.6% pesakit. 34.5% dikategorikan sebagai subkumpulan "germinal centre like (GCB)" dan 65.5% dikategorikan sebagai subkumpulan "non germinal centre like (non-GCB) subgroup". Antara ciri-ciri klinikopatologikal, "double/triple protein lymphoma" telah dikaitkan dengan ketara dengan peningkatan kadar LDH (p=0.018), skor IPI (p=0.003), penilaian Ann Arbor (p=0.011) dan kadar respon penuh (p=0.011).

**Kesimpulan:** "Double/triple protein lymphoma" telah berkait rapat dengan faktor risiko klinikal yang lebih buruk. Oleh itu, analisis ekspresi protein MYC, BCL2 dan BCL6 menggunakan immunohistokimia adalah pendekatan yang pantas dan murah untuk menstratifikasikan risiko pesakit pada masa didiagnosakan.

# ABSTRACT

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

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

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

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

# CHAPTER 1

## 1.0 INTRODUCTION

Cancer remains as one of the leading cause of death in the world. In 2008, it was estimated that about 12.7 million new cases and 7.6 million cancer deaths occurred worldwide with 56% of new cancer cases and 63% of the cancer deaths occurring in the less developed regions of the world (Ferlay *et al.*, 2010). In Malaysia, cancer is also one of the most common causes of death. According to the National Cancer Registry, a total of 18,219 new cancer cases comprises of 8,123 (44.6%) males and 10,096 (55.4%) females were diagnosed and registered in 2007 (Zainal Ariffin and Nor Saleha, 2011).

Lymphoma is one of ten leading cancers among population of Malaysia. Other cancers are breast, colorectal, lung, nasopharynx, cervix, leukaemia, ovary, stomach and liver. In 2007, a total of 776 new cases of lymphoma were reported in Malaysia comprising of 448 males and 328 females (Zainal Ariffin and Nor Saleha, 2011). The lymphoma ranked sixth most common cancer among Malaysian regardless of sex, the sixth most common cancer among males and eighth most common cancer among females. The incidence of lymphoma was slightly higher among males compared to females. Chinese were found to have higher incidence rate compared to Malay and Indian. While in Kelantan, lymphoma is the fourth most common cancer type among male comprise of 36 cases (8.33%) (Zainal Ariffin and Nor Saleha, 2011).

Lymphoma represents a combination of both Hogkin lymphomas (HL) and Non-Hodgkin's Lymphoma (NHL). NHL is the fifth leading type of new cancer cases among men and women, accounting for 4-5% of new cancer cases and 3% of cancer death among men and the sixth among women in the United States (Jemal *et al.*, 2008). The annual incidence of NHL is estimated to be 15–20 cases/100,000 in Europe and USA (Fisher and Fisher, 2004).

Classifying NHL can be quite confusing because there are so many types, therefore several different classification systems have been used before such as National Cancer Institute's Working Formulation (IWF) (Rosenberg, 1982) and Revised European-American Classification of Lymphoid Neoplasms (REAL) (Harris *et al.*, 1994).

The most recent system used is the World Health Organization (WHO) classification. The WHO classification first introduced in 2001 (Jaffe, 2001) and updated in 2008 (Swerdllow *et al.*, 2008) which based on the principles initially defined in the Revised European-American Classification of Lymphoid Neoplasms (REAL). This classification divides Non Hodgkin Lymphoma into two groups: those of B-cell origin and those of T-cell/natural killer (NK) cell origin. According to 2008 WHO classification, Diffuse Large B-cell Lymphoma (DLBCL) was classified under mature B-cell neoplasm (Swerdllow *et al.*, 2008) . Mature B cell neoplasm comprise over 90% of lymphoid neoplasm worldwide (Armitage and Weisenburger, 1998).

The aetiology of DLBCL remains unknown. In the 2008 WHO Classification, detailed discussion in the following morphological, biological and clinical studies have allowed the subdivision of DLBCL into morphological variants, molecular and immunophenotypic subgroups and distinct disease entities (Swerdllow *et al.*, 2008). Therefore, it is not surprise that many reseachers and scientists have dedicated their time and money in regards to studies, experiments and reviews to understand more about DLBCL.

The prognostic factors of DLBCL are still the hot issues for researchers. Prognostic factors in DLBCL can be divided into those related primarily to the patient (e.g. age and performance status), to the tumour itself (e.g. stage, tumour burden, proliferating fraction, extranodal involvement), to aggressiveness indicators (e.g. LDH serum level, 2-microglobulin levels, proliferating fraction), and to the therapeutic strategy (Martelli *et al.*, 2013). International prognostic index (IPI) based on clinical parameters is widely used for risk stratification but might be not reliable to predict the outcome in individual patients because of biological diversity of the disease (Lossos and Morgensztern, 2006).

According to gene expression profile (GEP), DLBCL can be divided into germinal center B-cell (GCB), activated B-cell (ABC) and unclassified subtypes (Alizadeh *et al.*, 2000; Rosenwald *et al.*, 2002; Wright *et al.*, 2003). These molecular subtypes are associated with different outcomes, even after the introduction of immunochemotherapy (Fu *et al.*, 2008; Lenz *et al.*, 2008). GEP is not availabe in most clinical laboratories, therefore different approached or algorithms with small panels of biomarker have been developed to translate the robust information from molecular studies into a routine

clinical platform (Colomo, 2003; Hans *et al.*, 2004; Muris *et al.*, 2006; Choi *et al.*, 2009; Meyer *et al.*, 2010).

Double-hit (DH) lymphomas are intriguing subset that is defined by a chromosomal breakpoint affecting the MYC/8q24 locus in combination with another recurrent breakpoint. Most commonly is MYC+/BCL2+. MYC+/BCL6+ DH lymphomas are relatively rare, and most of these cases represent BCL2+/BCL6+/MYC+ triple-hit lymphoma (Aukema *et al.*, 2011). Notably, DLBCL with translocation of both MYC and BCL2 is characterized by poor response to standard therapy and has an aggressive clinical course (Snuderl *et al.*, 2010; Johnson *et al.*, 2012).

To date, there has no tissue study of classifying pre-treatment DLBCL into GCB and ABC, and more of the double-hit/triple-hit, in correlation with the prognosis in DLBCL cases diagnosed in Hospital Universiti Sains Malaysia. From this study, it is hoped that it will be a starting point of using immunohistochemistry to classify DLBCL into germinal center B-cell (GCB) or activated B-cell (ABC) group, followed by testing of double/triple-hit when the diseases is resistant to the first line treatment.

# CHAPTER 2

## 2.0 LITERATURE REVIEW

## 2.1 DEFINITION AND INCIDENCE OF DIFFUSE LARGE B-CELL LYMPHOMA

Diffuse Large B-cell Lymphoma (DLBCL) is a neoplasm of large B lymphoid cells with nuclear size equal to or exceeding nomal macrophage nuclei or more than twice the size of a normal lymphocyte, that has a diffuse growth pattern (Swerdllow *et al.*, 2008). DLBCL is the most common type of Non-Hodgkin Lymphoma in adults, accounting for 31% of all Non Hodgkin Lymphoma in Western Countries (Armitage, 1997; Swerdllow *et al.*, 2008). In Malaysia, higher proportion was reported of approximately 60% (Chai *et al.*, 1999; Peh *et al.*, 2003).

The median age is in 7th decade although other types of aggressive Non-Hodgkin lymphoma (NHL) present at a lower median age, as for instance Burkitt lymphoma and primary mediastinal lymphoma (Smith *et al.*, 2011). DLBCL is the common lymphoid malignancy in adults (Armitage, 1997) but it may also occur in children and young adults. It is slightly common in males than in females. The probability of having DLBCL increases with age, from 0.13% and 0.09% before the age of 29 to 1.77% and 1.4% after the age of 70 in men and women, respectively (Sarkozy and Coiffier, 2013).

The aetiology of DLBCL remains unknown. It may arise as primary or *de novo,* or may results from a transformation of an indolent lymphoma eg chronic lymphocytic leukaemia/small lymphocytic leukaemia (CLL/SLL), follicular lymphoma, marginal zone lymphoma or nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) (Swerdllow *et al.*, 2008).

## 2.2 THE CLINICAL FEATURES AND MORPHOLOGY OF DIFFUSE LARGE B-CELL LYMPHOMA

The clinical presentation of DLBCL is diverse and depends on the site of disease involvement. DLBCL can develop at either nodal or extra-nodal sites. Extranodal involvement is common and is seen in up to one-third of patients at the time of diagnosis (López-Guillermo *et al.*, 2005). The most common extranodal presentation is in the gastrointestinal tract (stomach and ileocoecal region) but virtually any extranodal location may be a primary site. Other common sites of extranodal presentation include bone, testis, spleen, Waldeyer ring, salivary gland, thyroid, liver, kidney and adrenal gland. DLBCL is an aggressive disease, with the tumor showing a rapid growth rate, and patients present with rapid enlarging tumor masses infiltrating tissues or obstructing other organs, resulting in other symptoms (Swerdllow *et al.*, 2008).

Most Diffuse Large B-cell lymphoma (DLBCL) patients are asymptomatic but when symptoms are present they are highly dependent on the site of involvement (Armitage, 1997). Diffuse Large B-cell lymphoma (DLBCL) patients often present with B-symptoms including fever (≥38.5◦C), nights sweats, and weight loss (≥10% of the body

weight over 6 months) (Fameli-Pavlaki, 2005). Other symptoms reported by lymphoma patients include pruritus, fatigue, abdominal pain (enlarged spleen, liver, or bulky lymph node mass), bone pain (bone destruction or diffuse marrow involvement), neuropathic pain (nerve root compression, nerve infiltration, meningeal involvement, cord compression), and back pain (retroperitoneal lymph node involvement) (Armitage and Weisenburger, 1998).

DLBCL has a diverse cytomorphology. In DLCBL not otherwise specified (NOS), three common and additional minor morphological variants have been recognized which are centroblastic, immunoblastic, anaplastic variant and rare mophologic variant (Swerdllow *et al.*, 2008). DLBCL NOS usually consists of a mixture of centroblasts and immunoblasts that grow diffusely, partly or completely effacing the normal structure of the involved organ(s) (Martelli *et al.*, 2013).

Centroblasts are defined as medium sized to large lymphoid cells with a central oval to round nucleus, vesicular nuclei containing finely dispersed chromatin, two to four nuclear membrane bound nucleoli, and usually scanty and amphophilic to basophilic cytoplasm. Immunoblasts have a single centrally located nucleolus and an appreciable amount of basophilic cytoplasm with a more or less pronounced Golgi area. Immunoblasts may show aspect of plasmacytoid differentiation. Clinical and/or immunophenotypic findings may be essential in differentiating this variant from extramedullary involvement by a plasmablastic lymphoma or an immature plasma cell myeloma. Other morphologic variants are anaplastic large cell which characterized by large to very large round, oval or polygonal cells with bizzare pleomorphic nuclei that

resemble, at least in part, Hodgkin and/or Reed Sternberg cells, and may resemble tumour cells of anaplastic large cell lymphoma (Swerdllow *et al.*, 2008). The cells may show intrasinusoidal diffusion and cohesive growth pattern and may mimic undifferentiated carcinoma. On rare occasions, DLBCL with myxoid or fibrillary features or even consisting of spindle-shaped or signet-ring-like cells are encountered that represent a diagnostic challenge for the pathologist. All variants may be admixed with a high number of reactive T-cells and/or histiocytes. These cases should not be categorized as T-cell/histiocyte-rich large B-cell lymphoma as long they do not fullfil all the criteria of this T-cell/histiocyte-rich large B-cell lymphoma subtype (Swerdllow *et al.*, 2008; Martelli *et al.*, 2013).

## 2.3 CLASSIFICATION OF DIFFUSE LARGE B-CELL LYMPHOMA

Diffuse large B-cell lymphoma (DLBCL) encompasses a biologically and clinically diverse set of diseases (Alizadeh *et al.*, 2000). Based on morphological, biological and clinical studies, DLBCL has been subdivided into morphological variants, molecular and immunophenotypic subgroups and distinct disease entities. However, a large number of cases still remain biologically heterogeneous, for which there are no clear and accepted criteria for subclassification: these are collectively termed Diffuse Large B-cell lymphoma (DLBCL), not otherwise specified (NOS).

The most recent classification used for Diffuse Large B-cell lymphoma (DLBCL) is the World Health Organization (WHO) classification. Prior to that, there have been many other classifications for lymphoma, such as National Cancer Institute's Working

Formulation (IWF) (Rosenberg, 1982) and Revised European-American Classification of Lymphoid Neoplasms (REAL) (Harris *et al.*, 1994). The National Cancer Institute's Working Formulation (IWF), originally proposed in 1982, classified and grouped lymphomas by morphology and clinical behavior (ie, low, intermediate, or high grade) with 10 subgroups labeled A to J. In 1994, the Revised European-American Lymphoma (REAL) classification attempted to apply immunophenotypic and genetic features in identifying distinct clinicopathologic NHL entities. This classification divides NHL into two groups: those of B-cell origin and those of T-cell/natural killer (NK)–cell origin.

The World Health Organization (WHO) classification, first introduced in 2001 and updated in 2008, further elaborates upon the Revised European-American Lymphoma REAL approach. According to fourth edition of the WHO Classification of Haematopoietic and Lymphoid Tumours 2008, they have been subdivided into four categories, some of which not quoted in previous schemes: (a) DLBCL – not otherwise specified (DLBCL, NOS), (b) DLBCL subtypes (c) Other large B-cells d) Borderline cases. Each category includes morphologic and/or clinico-pathologic variants that make the organization of these neoplasms quite complex (Table 2.1). Identifying distinctive subgroups within the DLBCL category can assist with prognostication and therapeutic strategy (Visco *et al.*, 2013).

**TABLE 2.1: Diffuse large B-cell lymphoma: variant, subgroup and subtypes/entities. (Source: Swerdllow et al., 2008).**

| |
|---|
| **Diffuse large B-cell lymphoma (DLBCL), Not Otherwise Specified (NOS)**<br>Common Morphologic variants<br>Centroblastic<br>Immunoblastic<br>Anaplastic<br>Rare morphologic variant<br>Molecular subgroups<br>Germinal centre B-cell like (GCB)<br>Activated B-cell-like (ABC)<br>Immunohistochemical subgroup<br>CD5-positive DLBCL<br>Germinal centre B-cell-like (GCB)<br>Non-germinal centre B-cell-like (non-GCB) |
| **Diffuse large B-cell lymphoma subtypes**<br>T-cell/histiocyte rich large B-cell lymphoma<br>Primary DLBCL of the CNS<br>Primary cutaneous DLBCL, leg type<br>EBV positive DLBCL of the elderly |
| **Other lymphoma of large B cells**<br>Primary mediastinal (thymic) large B cell lymphoma<br>Intravascular large B-cell lymphoma<br>DLBCL associated with chronic inflammation<br>Lymphomatoid granulomatosis<br>ALK positive large B-cell lymphoma<br>Plasmablastic lymphoma<br>Large B-cell lymphoma arising in HHV8 associated with multicentric Castleman disease<br>Primary effusion lymphoma |
| **Borderline cases**<br>B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma<br>B-cell lymphoma, unclassifiable, with features intermediate between diffuse and large B-cell lymphoma and classical Hodgkin lymphoma |

## 2.4 MOLECULAR CLASSIFICATION OF DIFFUSE LARGE B-CELL LYMPHOMA

A cell of origin (COO) model based on gene expression profile (GEP) by using a cDNA microarray was developed to classify Diffuse Large B-cell lymphoma (DLBCL) into three prognostically significant subgroups; germinal center B-cell-like (GCB), activated B-cell-like (ABC) and unclassified subtypes which are believed to represent lymphomas arising from different stages of lymphoid differentiation (Figure 2.1) (Alizadeh *et al.*, 2000; Rosenwald *et al.*, 2002; Wright *et al.*, 2003).

The GCB and ABC subtypes have unique gene expression signatures. ABC subtypes was characterized by constitutive NF-κB activation, whilst the GCB is associated with BCL2 gene rearrangement t(14;18)(q32;q21) (Huang *et al.*, 2002; Iqbal *et al.*, 2004). The rearrangement of BCL6, expressed by germinal centre B- cells, is also important for GCB (Martelli *et al.*, 2013).

Currently it is impractical to perform microaaray analysis on every patient with DLBCL. Because of this, various immunohistochemical algorithms have been developed to predict the cells of origin and/or survival including Hans, Modified Hans, Choi, Modified Choi, Muris, Nyman and Tally algorithm, (Figure 2.2) (Colomo, 2003; Hans *et al.*, 2004; Muris *et al.*, 2006; Choi *et al.*, 2009; Meyer *et al.*, 2010). These algorithms use different combinations of antibodies eg CD10, BCL6 and MUM1/IRF4, GCET1, FOXP1 and LMO2 to detect germinal center or activated B-cell–related proteins.

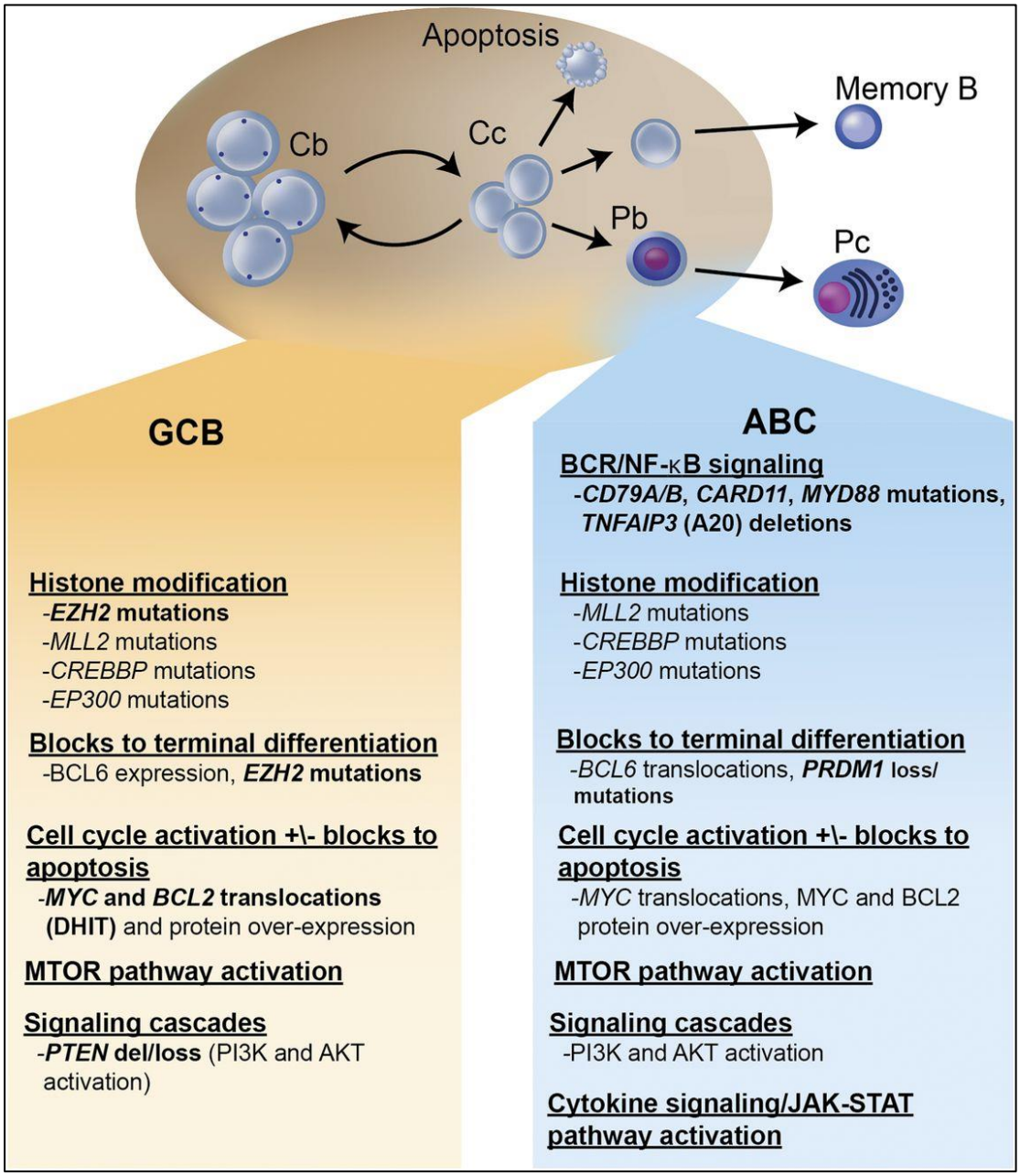


**Figure 2.1. Key oncogenic pathways in DLBCL. (Souce: Sehn and Gascoyne, 2015).**

**Note: The 2 major molecular subtypes of DLBCL are shown: the GCB and the ABC type. Both tumors arise from stages of differentiation reminiscent of germinal center B cells. The GCB subtype arises from centroblasts, whereas the ABC subtype arises from a plasmablastic cell just prior to germinal center exit. The main oncogenic pathways are listed, as well as the recurrent mutations, gains and losses of genetic material, and characteristic translocations that underlie these pathway perturbations. In bold are oncogenic mechanisms that are preferentially found in 1 molecular subtype. Cb, centroblast; Cc, centrocyte; Pb, plasmablast; Pc, plasma cell; DHIT, double-hit lymphoma; del, deletion; BCR, B-cell receptor.**

The results of the algorithms developed by Hans et al (2004) and Choi et al (2009) have correlated well with the corresponding GEP results and have also demonstrated clear survival differences between the germinal center B-cell-like (GCB) and non- germinal center B-cell-like (non-GCB) Diffuse Large B-cell lymphoma groups (Hans *et al.*, 2004; Choi *et al.*, 2009). The results of algorithms developed by other authors have not been compared with the corresponding GEP results and rely predominantly on survival differences between the immunophenotypic groups (Muris *et al.*, 2006; Natkunam *et al.*, 2008; Nyman *et al.*, 2009).

Meyer et al. (2010) found the Choi algorithm and Hans algorithm had high concordance with the microarray results. In this study, modifications of the Choi and Hans algorithms for ease of use by removing the BCL6 immunostain also showed high concordance with the microarray results. Immunostain for BCL6 are technically difficult to perform and result in difficulties in interpretation (De Jong *et al.*, 2007). However the most extensively used and validated immunohistochemical model is the Hans classifier (Hans *et al.*, 2004).

According to Hans classification, the cases were classified into 2 groups: Germinal Center B-cell-like (GCB) and non-Germinal Center B-cell-like (non-GCB) by using CD10, BCL6 and MUM1. BCL6 and CD10 are markers of germinal center B cells (Flenghi *et al.*, 1996; Dogan *et al.*, 2000; Falini *et al.*, 2000) whereas MUM1 is expressed in plasma cells and the later stages of B-cell development (Falini *et al.*, 2000), and it is associated with the ABC group in gene expression profiling studies

(Rosenwald *et al.*, 2002). The cases were assigned to the GCB group if CD10 alone was positive or if both BCL6 and CD10 were positive. If both BCL6 and CD10 were negative, the case was assigned to the non-GCB subgroup. If BCL6 was positive and CD10 was negative, the expression of MUM1 determined the group: if MUM1 was negative, the case was assigned to the GCB group; if MUM1 was positive, the case was assigned to the non-GCB group.

Microarray analysis has shown that patient with DLBCL expressing a GEP of GCB have a better and longer survival than those with a GEP of ABC (Alizadeh *et al.*, 2000; Lenz *et al.*, 2008).

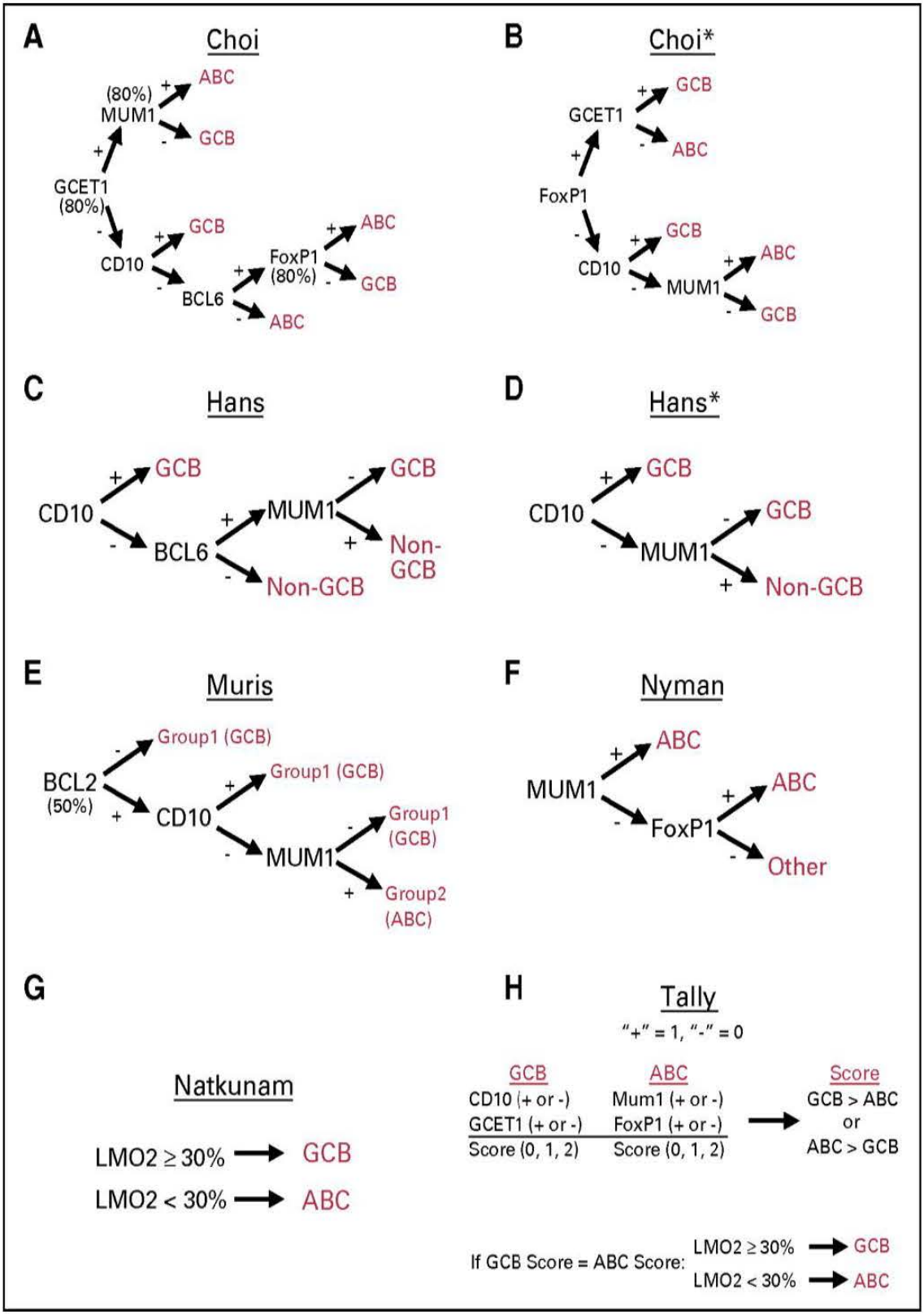


**Figure 2.2: Immunohistochemical algorithm used in Meyer et al., 2010. (Source: Meyer et al., 2010).**

## 2.5 PRE-TREATMENT EVALUATION, STAGING AND PROGNOSTIC FACTORS OF DIFFUSE LARGE B-CELL LYMPHOMA

The diagnosis of Diffuse Large B-cell lymphoma (DLBCL) includes an excision biopsy to assess the tumor architecture and providing adequate specimen for phenotypic and molecular studies. Needle core biopsy is only recommended in patients in whom a surgical approach is not possible or advised. Fine needle biopsy is not recommended (Parker *et al.*, 2010). Once the diagnosis has been established the first critical step is the pre-treatment evaluation and staging.

In pre-treatment evaluation, a complete history and physical examination are the two important components. Physical examination includes evaluation of all lymph nodes enlargement, site and size of all abnormal lymph nodes, evaluation of the presence or absence of hepatosplenomegaly, inspection of the skin, and detection of other palpable masses. The assessment of the extranodal involvement is important because extranodal involvement affects the prognosis of patients who are undergoing R-CHOP therapy for DLBCL (Takahashi *et al.*, 2012). The presence or absence of B symptoms and other symptoms that may show specific sites of involvement should be noted (Martelli *et al.*, 2013).

An assessment of performance status is also important in all patients, and especially for those who are entering into clinical research trials. There are various scoring systems used. The most generally used are Eastern Cooperative Oncology Group (ECOG) score

(Oken *et al.*, 1982) (Table 2.2) and Karnofsky score (Karnofsky *et al.*, 1948). In our study, we used ECOG score in view of its simplicity over the Karnofsky score.

**Table 2.2: Eastern Cooperative Oncology Group (ECOG) score. (Source: Oken et al., 1982).**

| **ECOG PERFORMANCE STATUS** | |
|---|---|
| **GRADE** | **ECOG** |
| 0 | Asymptomatic (Fully active, able to carry on all predisease activities without restriction) |
| 1 | Symptomatic but completely ambulatory (Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature. For example, light housework, office work) |
| 2 | Symptomatic, <50% in bed during the day (Ambulatory and capable of all self care but unable to carry out any work activities. Up and about more than 50% of waking hours) |
| 3 | Symptomatic, >50% in bed, but not bedbound (Capable of only limited self-care, confined to bed or chair 50% or more of waking hours) |
| 4 | Bedbound (Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair) |
| 5 | Death |

Laboratory investigations that should be routinely performed in DLBCL patients include a complete blood count to assess bone marrow reserves and a white blood cell differential with careful examination of the peripheral blood to look for the presence of circulating lymphoma cells. Serum chemistry should include an assessment of hepatic and renal function. Lactate dehydrogenase (LDH) is an important indicator of tumour activity and is included as prognostic factors in the International Prognostic Index

(Shipp *et al.*, 1993). Based on our centre, Hospital Universiti Sains Malaysia, the normal range of LDH level is < 480 U/L. The uric acid level may predict patients at increased risk for urate nephropathy. A test for a complete assessment of HIV, HBV, and HCV should also be performed in all patients. Where treatment is considered, bone marrow biopsy with or without aspirate is essential (Martelli *et al.*, 2013).

The standard staging system used for Diffuse Large B-cell lymphoma (DLBCL) is Ann-Arbor classification system which was proposed at the Ann Arbor Conference in 1971 (Carbone *et al.*, 1971) and the Cotswolds modification (Lister *et al.*, 1989) (Table 2.3). This staging system reflects the number of sites of involvement and their relation to the diaphragm, the existence of B symptoms (fevers >38 $^{\circ}$C for at least three consecutive days, night sweats, body weight loss >10% during the 6 months prior to diagnosis) and the presence of extranodal disease.

**Table 2.3: Ann Arbor Classification and the Cotswold Modifications. (Source: Armitage, 2005).**

| STAGE | FEATURES |
|---|---|
| I | Involvement of a single lymph node region or lymphoid structure (eg, spleen, thymus, Waldeyer's ring) |
| II | Involvement of two or more lymph node regions on the same side of the diaphragm |
| III | Involvement of lymph regions or structures on both sides of the diaphragm |
| IV | Involvement of extranodal site(s) beyond that designated E |
| For all stages | |
| A | No symptoms |
| B | Fever (38$^{o}$C), drenching sweats, weight loss (10% body weight over 6 months) |
| For Stages I to III<br>E | Involvement of a single, extranodal site contiguous or proximal to known nodal site |
| Cotswold modifications | Massive mediastinal disease has been defined by the Cotswold meeting as a thoracic ratio of maximun transverse mass diameter greater than or equal to 33% of the internal transverse thoracic diameter measured at the T5/6 intervertebral disc level on chest radiography. |
| | The number of anatomic regions involved should be indicated by a subscript (eg, II3) |
| | Stage III may be subdivided into: III1, with or without plenic, hilar, celiac, or portal nodes;III2, with para-aortic, iliac, mesenteric nodes |
| | Staging should be identified as clinical stage (CS) or pathologic stage (PS) |
| | A new category of response to therapy, unconfirmed/uncertain complete remission (CR) can be introduced because of the persistent radiologic abnormalities of uncertain significance |

The Ann Arbor staging system does not adequately provide prognostic information for many subtypes of Non-Hodgkin Lymphoma and is far from optimal making treatment decisions (Armitage, 1997). Most non-Hodgkin Lymphomas are not localized and the ability to better assess the majority of patients for treatment decisions and to stratify patients in clinical trials is extremely important. Thus, in 1993, the International Non-Hodgkin Lymphoma Prognostic Index (IPI) was published (Shipp *et al.*, 1993) to aid in predicting the prognosis of patients with aggressive non-Hodgkin's lymphoma (Table 2.4). This was the result based on an international collaboration that involved 2031 patients with aggressive non-Hodgkin Lymphoma, treated with an anthracycline-based combination chemotherapy regimen. Although originally intended for the use in patients with aggressive lymphomas, this prognostic index has utility for all types of non-Hodgkin Lymphoma and has been widely applied. Results of the IPI study showed that five factors were roughly equal in power in predicting treatment outcome. These included age (≤ 60 years *vs* > 60 years), lactate dehydrogenase (LDH) value (≤ upper limit of normal [ULN] *vs* > ULN), performance status (ECOG) 0, 1 *vs* > 1), Ann Arbor stage (I/II *vs* III/IV), and the number of extranodal involvements (0, 1 *vs* > 1). The original publication suggested the patients be lumped into groups with a low risk (ie, score of 0 or 1), low intermediate risk (score of 2), a high intermediate group (score of 3), and a high risk (score of 4 or 5). An age-adjusted model or known as age-adjusted IPI (aaIPI) was also constructed for younger patients aged ≤60 years (Table 2.4).

In the original study and in previous experience, there is a highly significant impact on chances to achieve a remission, remain in remission, and overall survival based on the IPI score. For example, patients in the low-risk group in the original publication had an 87% complete remission rate and an overall survival of 73% at 5 years versus a 44%

complete remission rate and 26% survival at 5 years in the high- risk group (Shipp *et al.*, 1993).

**Table 2.4: International Prognositic index (IPI). (Source: Martelli et al., 2013)**

| **IPI** | | **aa-IPI** | |
|---|---|---|---|
| **Risk group** | **IPI factors** | **Risk group** | **IPI factors** |
| Low | 0 or 1 | Low | 0 |
| Low intermediate | 2 | Low intermediate | 1 |
| High intermediate | 3 | High intermediate | 2 |
| High | 4 or 5 | High | 3 |
| **IPI Factors** | | | |
| Older than 60 years of age (not used for aa-IPI) | | | |
| Disease stage III/IV | | | |
| Lactate dehydrogenase level elevated | | | |
| ECOG performance score ≥ 2 | | | |
| Extranodal disease > 1 site (not used for aa-IPI) | | | |

18Fluorodeoxyglucose Positron Emission Tomography (PET) scan is a noninvasive, 3-dimensional imaging modality that has become a standard procedure both for staging and response assessment. Many studies showed that PET scan at the end of treatment is highly predictive of progression-free survival (PFS) and overall survival (OS) in aggressive lymphomas with or without residual masses detected with CT scan (Juweid *et al.*, 2007; Seam *et al.*, 2007; Cheson, 2009). PET scan is able to distinguish between lymphoma and necrosis or fibrosis in residual masses. The combination of International Workshop Criteria (IWC) and PET were evaluated in a retrospective analysis of 54 patients with Non-Hodgkin Lymphoma. Based on Juweid *et al*. (2007) study, the International Harmonization Project has provided new recommendations for response criteria for aggressive malignant lymphomas, incorporating PET into the definition of response at the end of treatment. DLBCL arisen in particular extranodal sites require further "specific" staging studies that vary somewhat by site. This is important in primary CNS Lymphoma (Ferreri and Reni, 2007) testicular DLBCL (Zucca *et al.*,

2003) and gastric DLBCL (Ferreri and Montalbán, 2007).

## 2.6 DOUBLE OR TRIPLE HIT LYMPHOMA

Double hit (DH) or also referred as “Dual Hit” lymphomas are intriguing subset that is defined by a chromosomal breakpoint affecting the MYC/8q24 locus in combination with another recurrent breakpoint. Most commonly is MYC+/BCL2+ which involving the BCL2 at t(14; 18)(q32;q21). BCL6+/MYC+ DH lymphomas are relatively rare, and most of these cases represent BCL2+/BCL6+/MYC+ are of triple-hit lymphomas (Aukema *et al.*, 2011).

Approximately a third of DLBCL cases harbor a BCL2 or BCL6 rearrangement, MYC rearrangements are less common and detected by fluorescent in situ hybridization (FISH) in 5 to 14 % of cases (Iqbal *et al.*, 2004; Barrans *et al.*, 2010). Green et al. (2012) reported approximately 5% of DLBCL are double-hit lymphomas, which involved translocation of both MYC and BCL2.

Majority cases with DH lymphoma having a germinal center phenotype and expression of BCL2. Patients with DH lymphomas often presented with poor prognostic parameters, including elevated LDH, bone marrow and CNS involvement, and a high IPI score (Aukema *et al.*, 2011).

DLBCL cases that harbor a BCL2 or BCL6 rearrangement alone have not been associated with a worse outcome compared to cases without these rearrangements. However, several reports have demonstrated that a MYC rearrangement confers a worse prognosis following treatment with CHOP or R- CHOP (Savage *et al.,* 2016). DLBCL with translocation of both MYC and BCL2 (double-hit lymphoma) has poor response to standard therapy and has an aggressive clinical course (Johnson *et al.,* 2009; Snuderl *et al.,* 2010; Li *et al.,* 2012).

More recently, others have extended the concept of MYC/BCL2 double-hit lymphoma by assessing for MYC and BCL2 protein expression by immunohistochemistry (IHC), the logic being that protein expression, regardless of mechanisms, may have prognostic significance (Green *et al.*, 2012; Johnson *et al.*, 2012; Kluk *et al.*, 2012). These studies were possible because of the recent availability of anti-MYC antibodies and anti-BCL2 antibodies which suitable for IHC staining in paraffin-embedded tissues. These IHC markers are easier to perform, especially in centres without the facilities of fluorescent in situ hybridization (FISH) or other molecular techniques. This technique also can identify DLBCL patients who have a high likelihood of demonstrating an unsatisfactory response to standard treatment and who could be candidates for novel therapeutic strategies (Green *et al.*, 2012).

In two studies, Green et al (2012) and Johnson et al (2012) showed that DLBCL patients with MYC/BCL2 coexpression, with or without MYC or BCL2 gene rearrangements, have a poorer prognosis with inferior overall survival and progression-free survival. However most of the cases with double protein expressor or double

protein lymphoma (DPL) (overexpression MYC and BCL2) are without translocation. Unlike classic double-hit DLBCL, double protein expressor DLBCL is much more prevalent (~30% vs 5% of DLBCL) and two-thirds of patients are ABC subtype or non-GCB subtype (Figure 2.3), depending on the platform used to establish cell of origin (COO) (Green *et al.*, 2012; Johnson *et al.*, 2012; Horn *et al.*, 2013; Hu *et al.*, 2013).

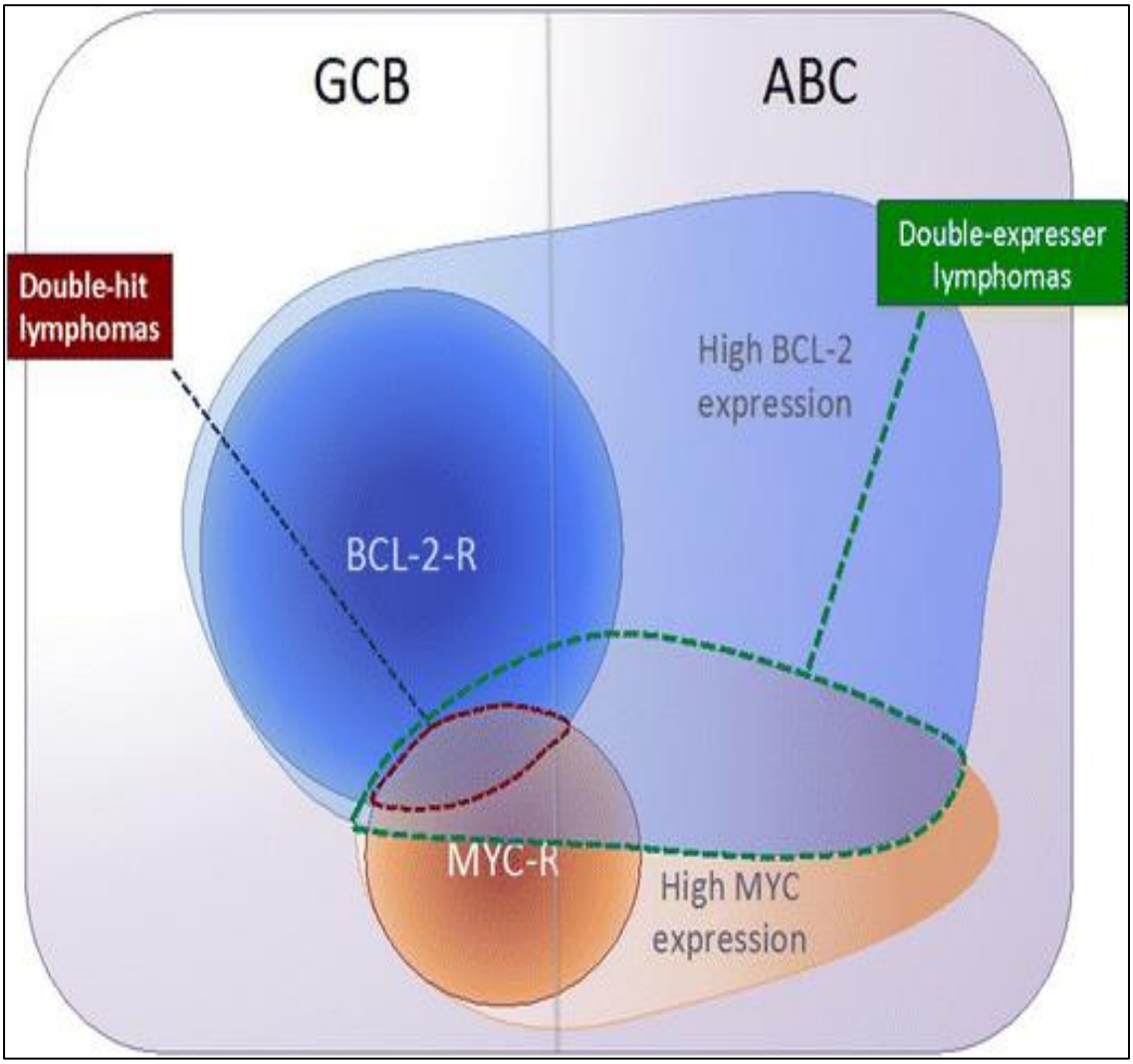


**FIGURE 2.3: The relationship of cell of origin and high BCL2 and MYC expression in Diffuse Large B-cell lymphoma (DLBCL). Most cases of double-hit lymphoma are of germinal center B cell (GCB) origin whereas most cases of double-expresser lymphomas, without any hits, are of activated B cell (ABC) origin. (Source: Dunleavy, 2015).**