MOLECULAR CHARACTERIZATION OF DIFFUSE LARGE B-CELL LYMPHOMA AND IT'S PROGNOSTIC SIGNIFICANCE

Thesis submitted to the Tamil Nadu Dr.M.G.R Medical University

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DOCTOR OF PHILOSOPHY

By

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DECLARATION

I declare that the thesis entitled "Molecular Characterization of Diffuse Large B-Cell Lymphoma and it's Prognostic significance" submitted by me for the degree of Doctor of Philosophy (Ph.D.) is the record of research work carried out by me during the period 2008-2013 under the guidance of Dr. T. Rajkumar, Professor and Head, Department of Molecular Oncology, Cancer Institute (WIA), and has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship, Titles in this University or any other University or other similar institution of Higher Learning.

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CERTIFICATE

I certify that the thesis entitled **"MOLECULAR CHARACTERIZATION OF DIFFUSE LARGE B-CELL LYMPHOMA AND IT'S PROGNOSTIC SIGNIFICANCE"** submitted by **Dr. S. Shirley**, is a research work done during 2008 - 2013 under my supervision and that the thesis has not previously formed the basis for the award to the candidate of any Degree, Diploma, Associateship, Fellowship or other similar title.

I also certify that this thesis represents complete independent work on the part of the candidate.

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CHAPTER 1

INTRODUCTION

Lymphomas are a diverse group of malignant neoplasms that arise from clonal expansion of neoplastic B and T lymphocytes derived from normal counterparts in the immune system. Lymphomas are broadly classified into Hodgkin's lymphoma and non Hodgkin's Lymphoma. The current World Health Organization (WHO) classification of lymphomas provides an up-to-date and biologically relevant approach to lymphomas by incorporating a variety of parameters (morphology, immunophenotypic findings, molecular genetic studies and cytogenetic features) and has become the gold standard.[1]

Non Hodgkin's Lymphoma (NHL) derive from mature B cells (85% of cases) and in a minority of cases from T cells. Mature B cell neoplasms constitute more than 90% of lymphoid neoplasms globally. The incidence of NHL varies across the world with the highest incidence rates in developed countries like Unites States, Australia, New Zealand and Europe. Though low incidence rates have been reported in Asia, it is one of the ten leading cancers in India. According to the Madras Metropolitan Tumour Registry data (MMTR), the Crude Incidence Rate (CIR) of NHL, for the year 2009 - 2010 is 4.8% of cancer cases in males and 3.1% of cancer cases in females. In a recent study published on the frequency and distribution of lymphoma types in a tertiary care hospital in South India, NHLs constituted 78.7%. Of these, B-cell neoplasms accounted for 78.6% and T-cell/natural killer (NK)-cell neoplasms 20.2% of the NHLs. The commonest subtype of NHL was diffuse large B-cell lymphoma (46.9%). [2] In another study published on the distribution pattern of NHLs in India, B- and T-cell NHLs constituted 79.3% and 18.8%

of cases. Diffuse large B-cell lymphoma (DLBCL) was the most common subtype.[3] A lower frequency of follicular lymphoma, marginal zone lymphoma and mantle cell lymphoma was noted compared to that observed in the developed countries, whereas a lower frequency of peripheral T-cell lymphoma - not otherwise specified and extranodal NK/T-cell lymphoma was seen compared to that in the other Asian countries.[3] In yet another large study, B-cell lymphomas formed 79.1% of the NHLs among which DLBCL was the most common subtype (34%). Follicular lymphomas, B-cell small lymphocytic lymphoma, mantle-cell lymphoma, and marginal zone B-cell lymphomas (including MALT lymphomas) amounted to 12.6%, 5.7%, 3.4%, and 8.2%, respectively. T-cell lymphomas formed 16.2% of the total among which T-cell lymphoblastic lymphoma, anaplastic large-cell lymphomas of T/null-cell type, and other nodal peripheral T-cell lymphomas accounted for 6%, 4.3%, and 2.9% of all cases, respectively. Therefore the distribution of NHL subtypes in India show important differences with those from the rest of the world. Follicular lymphoma and mantle-cell lymphoma are less common in India compared to Europe and the USA. Peripheral T-cell lymphomas and T/NK-cell lymphomas of nasal type, which are common in many other Asian countries, are also less prevalent.[4]

Diffuse large B-cell lymphoma is a neoplasm of large B lymphoid cells with nuclear size equal to or exceeding normal macrophage nuclei or more than twice the size of a normal lymphocyte with a diffuse growth pattern.[1] It is the most common lymphoma subtype and accounts for 30–40% of adult non-Hodgkin lymphoma. DLBCLs are aggressive B-cell lymphomas that are clinically, pathologically and genetically diverse, in part reflecting the functional diversity of the B-cell system. The diversity of DLBCL is

reflected in the current 2008 WHO classification that has subdivided DLBCL into morphological variants, molecular and immunophenotypical subgroups and distinct disease entities. The focus in recent years has been towards incorporation of clinical features, morphology, immunohistochemistry and ever evolving genetic data into the classification scheme. The 2008 WHO classification reflects this complexity with the addition of several new entities and variants.

Diffuse large B-cell lymphoma, not otherwise specified (NOS)

Common morphologic variants: centroblastic, immunoblastic, anaplastic

Rare morphologic variants

Molecular subgroups: Germinal centre B-cell-like (GCB), Activated B-cell-like (ABC)

Immunohistochemical subgroups: CD5-positive DLBCL, Germinal centre B-cell-like (GCB), non-germinal centre B-cell-like (GCB)

Diffuse large B-cell lymphoma subtypes

T-cell/histiocyte-rich large B-cell lymphoma

Primary DLBCL of the CNS

Primary cutaneous DLBCL, leg type

EBV positive DLBCL of the elderly

Other lymphomas of large B-cells

Primary mediastinal (thymic) large B-cell lymphoma

Intravascular large B-cell lymphoma

DLBCL associated with chronic inflammation

Lymphomatoid granulomatosis

ALK-positive large B-cell lymphoma

Plasmablastic lymphoma

Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease

Primary effusion lymphoma

Borderline cases

B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma

B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

Historically, lymphoma classification has been based on the presumed normal counterpart. Thus, some of the subtypes of diffuse large B-cell lymphomas reflect the putative cell of origin, e.g. mature B-cell vs. more differentiated plasma cells. However, adopting this methodology as the sole means of classification is perhaps inadequate or incomplete in defining certain subtypes of lymphoma, e.g. intravascular large B-cell lymphoma. It has been shown recently that biologically and clinically meaningful subgroup can be defined by gene expression profiling (GEP). The discovery of distinct subtypes by GEP has heralded a new era with a focus on pathways of transformation as well as a promise of more targeted therapies, directed at specific pathways.

CHAPTER 2

AIMS AND OBJECTIVES

2.1 AIMS

Approximately half of all patients with DLBCL can be cured by conventional CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisone) which remains the standard treatment. Nevertheless more than 50% of patients never attain remission and eventually die of the disease. It may be possible to identify subgroups of patients with a characteristic molecular profile associated with poor prognosis. Such high risk groups can be treated with more aggressive primary therapy or by alternative therapeutic strategies such as use of monoclonal antibodies and multimodal chemotherapy combinations. It is important to determine if such high risk groups can be identified based on readily available, low cost, immunohistochemistry based molecular profiling of DLBCL. This cost effective profiling of DLBCL may be important for a low resource country like India

Current attempts to evaluate prognosis in DLBCL is based on clinical parameters, most notably the International Prognostic Index (IPI) score.[5] This is based on readily available clinical pretreatment prognostic factors (age, performance status, stage, number of extranodal sites, serum lacatate dehydrogenase) and has been recognized as the most effective tool to define prognostic subgroups in DLBCL and for predicting outcome of patients with DLBCL. However stratification on the basis of these general clinical parameters ignores the inherent biologic heterogeneity that underlies DLBCLs. These biological differences not fully accounted for by the IPI, may substantially modify the clinical outcome.[6] Hence pathologic or biologic variables have been sought to provide the

correlates that underlie the clinical behaviour of these lymphomas. By combining pathologic prognostic factors with the IPI, prediction of patients with DLBCL can be improved and this method of risk stratification will be justifiable. The ultimate goal will be to identify groups of DLBCL patients based on molecular profile, clinical factors and associated key molecular markers (if any) driving tumour cell proliferation and survival. Identification of such groups of patients with pivotal molecular markers can be selected as candidate group for testing targeted therapies designed specifically against the incriminating molecules.

Numerous studies have been undertaken to find new markers that can identify patients who will not respond with current multiagent chemotherapy. This implies that there are biologic differences between lymphomas that will independently affect the clinical outcome. DLBCL seems to be the result of deregulation of multiple genes involved in the control of cell cycle, apoptosis, cell growth, DNA repair, ubiquitin degradation and others.

One of the most important issues in DLBCL is to refine the standard prognostic scores. Easily quantifiable biological parameters may provide prognostic information that supplements, compliments or may even supersede the clinically based IPI. More importantly our molecular understanding of DLBCL is rapidly evolving and with it the identification of many new proteins that may play an important role in determining prognosis. Gene Expression Profiling (GEP) has identified two main groups of DLBCL – Germinal Center B like (GCB) and Activated B like (ABC) subgroups. This has significantly altered the approach to the assessment of biomarkers in DLBCL. Striking differences in the outcome of patients belonging to these two groups of lymphomas have

been reported with the activated B-like lymphomas having a worse prognosis.[7,8] Future approaches to DLBCL management will use molecular signatures identified through GEP to provide prognostic information and to isolate therapeutic targets for DLBCL patients.[9] Although GEP technology is immensely powerful, it is not readily available in the clinical setting.[6] The requirement of fresh or frozen tissue precludes its suitability for routine clinical use. The subgrouping of DLBCL by immunohistochemistry (IHC) would be of great practical value. Moreover identification of poor-risk subgroups of DLBCL using immunohistochemical stains would have practical utility with regard to prognosis and therapeutic decisions.[10] Immunohistochemically Bcl-6 and CD10 have been most widely used as markers of the germinal center (GCB) phenotype and Mum1 as a marker of activated B like (ABC) phenotype.

Though there have been isolated reports on the expression pattern of bcl-2 family of proteins in indolent B-cell NHLs [11] there is no published data on the differentiation profile and prognostic biomarkers in DLBCL to the best of my knowledge. Though DLBCL is the most common subtype of B-cell NHL encountered in India with more number of cases as compared to the west, most of the published data on study of biomarkers in DLBCL are from the west. The differences in the relative proportions of NHL subtypes between developing countries like India and the rest of the world presumably arise from differences in environmental and genetic factors that influence lymphomagenesis.[12] This difference strongly supports the argument in favour of more research in developing countries like India in order to provide valuable insights into molecular profiling of DLBCLs and identify geographic differences if any. Hence this study aims at studying the expression patterns of various biomarkers, proliferative

activity and ploidy status in DLBCL for better risk stratification of our patients and identification of groups of patients who may benefit with early aggressive therapy and alternate therapeutic strategies.

Hence this study aims at studying the expression patterns of various biomarkers, cell cycle and ploidy status in DLBCL for better stratification of our patients who might benefit from alternative therapeutic strategies.

2.2 OBJECTIVES

1. To classify DLBCL into Germinal Center B like (GCB) and Activated B like (ABC) subtypes using Bcl-6, CD10, Mum1 antibodies and determine the clinical significance and prognostic value of the differentiation profile.

2. To evaluate the protein expression patterns of cell cycle regulatory proteins (p16,p14, p21,p27,CyclinD2, CyclinE, CyclinA, CyclinB1), tumour suppressor proteins (p53, Blimp 1), oncoprotein mdm2, proliferative marker (Ki 67), apoptosis-regulating proteins (Bcl-2, Bax) and NF-kB in patients with DLBCL and assess the relationship of these proteins to overall survival.

3. To analyze cell cycle and DNA content (ploidy) within tumour cells from paraffin embedded tissues and evaluate additional prognostic information beyond that derived from the clinically based IPI.

4. To perform P53 mutation analysis and correlate it with p53 protein expression.

CHAPTER 3

REVIEW OF LITERATURE

Diffuse large B-cell lymphoma is a heterogenous clinicopathologic entity whose molecular pathogenesis is complex with multihit process culminating in the development of a malignant clone of germinal or post germinal center B-cell origin. While some mechanisms in the molecular pathogenesis of DLBCL is known, many still remain unknown. The majority of DLBCLs have increased expression of BCL6 gene leading to repression of target genes including the P53 tumour suppressor gene. Loss of P53 function results in inability of cells to undergo apoptosis in response to DNA damage.

BCL6 (B-cell lymphoma-6) is a transcriptional repressor which mediates its biological effects through downregulation of over 500 direct target genes mainly involved in control of cell cycle, gene transcription, DNA damage sensing, protein ubiquitylation and chromatin structure.[13] Constitutive expression of BCL6 mediates lymphomagenesis through aberrant proliferation, survival and differentiation blockade.[14] BCL6 gene is normally expressed in B cells of the follicular germinal centers and it is necessary for GC formation. Bcl-6 represses genes involved in lymphocyte activation differentiation and apoptosis within the germinal center. Genetic alterations of this gene are frequently detected in DLBCL.[15] Bcl-6 protein, a zinc finger protein that functions as transcriptional repressor is selectively expressed by GCB cells in normal lymphoid tissues. Bcl-6 protein expression is a useful prognostic indicator in patients with DLBCL. Positive bcl-6 immunostaining has been associated with a favourable prognosis and is a predictor of improved survival.[16,10] BCL6 is being increasingly investigated as a candidate for

molecular targeted therapy using various BCL6 inhibiting molecular technoques. Unfortunately, DLBCL is a disease with complex multistep pathogenesis and hence even targeted therapy against the pivoyal molecule like BCL6 may not yield favourable response.[17]

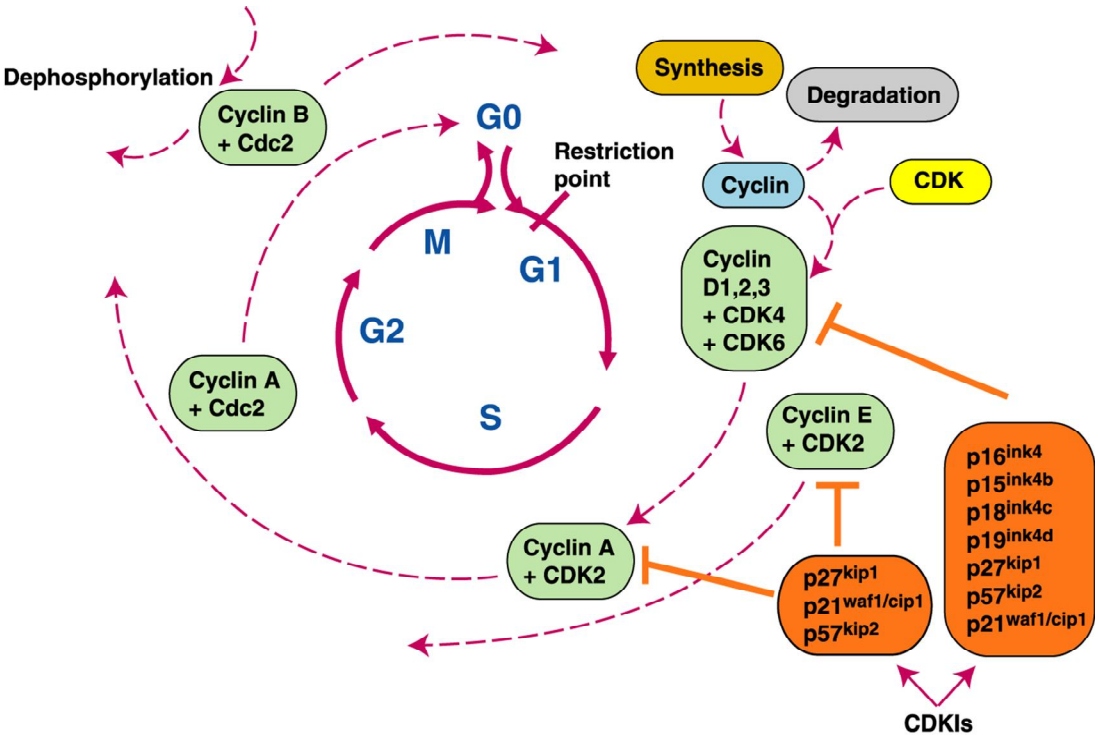
CD10 (Common Acute Lymphoblastic Leukemia Antigen – CALLA) is a membrane metalloproteinase that is detected in early lymphoid progenitors and in a variety of epithelial cells. In secondary lymphoid organs, CD10 expression is restricted to germinal centers of secondary follicle and thus serves as a marker of GCB-DLBCL. Expression of CD 10 is associated with better overall survival.[18,19]

MUM1 (Multiple myeloma oncogene 1) a novel member of the interferon regulatory factor (IRF) family of genes plays a role in the terminal phases of B-cell differentiation. In normal B-cells, MUM1 expression is thought to denote the final step of intra-germinal center B-cell differentiation and subsequent steps of maturation towards plasma cells. Mum1/IRF 4 protein was introduced to the panel of phenotypic markers available for characterization of B-cell differentiation, and is important in recognizing post-GC (ABC) DLBCLs. DLBCLs expressing MUM1 have poor clinical course and has been shown to strongly predict outcome.[18]

Determination of cell of origin is insufficient for accurate prediction of outcome in DLBCL. DLBCL may result from concurrent abnormalities in several pathways.[20] Particularly striking is the presence of multiple concurrent abnormalities in the genes and pathways in the control of cell cycle and apoptosis.

Regulation of cell cycle involves a large number of activating and inhibiting proteins commonly altered in many cancers **(Figure 1)**.

Figure 1: Cell Cycle



Adapted from: Shaw and Schwartz . Clin.Can. Res. 2001:7; 2168-2181

As proliferation of cells is essential for tumour growth, analysis of the cell cycle and its individual phases might give additional information on tumour progression and clinical behaviour. Alterations in cell cycle regulation most frequently involves tumour suppressor genes such as p53 and $p16^{INK4a}$. Lymphomas that acquire these alterations show an aggressive clinical behaviour and treatment resistance irrespective of their histologic grade.[21]

Cell cycle progression is mediated by the activation of a highly conserved family of protein kinases called the cyclin-dependent kinases (cdks). Activation of a cdk requires binding to a specific regulatory subunit termed a cyclin – so named because of their fluctuating levels through the cell cycle. To date atleast 15 cyclins have been described.

Extracellular stimuli elevate D-type cyclins (D1,D2,D3) which bind to and activate cdk4 and cdk6 and stimulate quiescent cells to enter the cell cycle or proliferating cells to continue proliferation. After elevation of D-type cyclins and activation of cdk4 or 6 in G1, cyclin E levels increase and bind to cdk2 in cell. The cyclin E/cdk2 complex regulate the transition from G1 to S phase. Cyclin A is thought to be involved in the regulation of S phase entry, and it is also important in G2 and M phases. The entry into mitosis from G2 is under the control of Cyclin B which associates with cdc2 (cdk1). Cyclin B is necessary for the activation of the cdk1 (cdc2) kinase at the onset of mitosis.

The expression of cyclin D2 is an independent predictor of poor overall survival. Cyclin D2 expression will be useful in designing a biological prognostic index for patients with DLBCL.[10,22,23]

Deregulation of cyclin E is a strong predictor of poor prognosis in some neoplastic diseases. CCNE expression assessment is easy on paraffin-embedded tissue. Cyclin E expression in DLBCLs has emerged as an independent predictive factor for standard CHOP treatment resistance. The high prognostic value of Cyclin E expression in DLBCL may be the basis for future prospective trials. High Cyclin E expression has been shown to be associated with poor prognosis.[24,25]

High expression of Cyclin A is seen in aneuploid and high grade B-NHLs and associated with a poor prognosis and survival.[26]

Cyclin B1 expression is an independent prognostic marker for poor outcome in diffuse large B-cell lymphoma. Its evaluation as a prognostic marker in the assessment of this entity is easily applicable in daily routine practice.[27]

Cyclin-dependent kinases (cdks) are regulated by a group of functionally related proteins called cyclin dependent kinase inhibitors (CDKI). The cdk inhibitors fall into two families. The INK 4 inhibitors and the Cip/Kip inhibitors. p16 ^{INK4A} and p19 ^{INK4D} belong to the INK 4 family of CDKI. p21 $^{Waf\ 1/Cip\ 1}$ and p27 ^{Kip1} belong to the Cip/Kip family members. The INK 4 family specifically inhibits cdk 4 and cdk 6 activity during the G1 phase of the cell cycle, while the Cip/Kip family can inhibit cdk activity during all phases of the cell cycle.

p16 $^{(INK4A)}$ binds to CDK 4 specifically and inhibits the activities of cyclin D and cdk4/6. p16, a tumour suppressor gene located on chromosome 9p21 is reported to be deleted in many cancers. It seems to be inactivated by genetic or epigenetic mechanisms in many DLBCL cases and has been shown to be an adverse prognostic marker.[28] p16 inactivation leads to transformation of low-growth fraction lymphomas into their aggressive variants.[21]

p14 $^{(INK4D)}$ blocks MDM2 induced p53 degradation. p14/ARF functions as an upstream regulator of the p53 pathway by stabilizing p53 through sequestering MDM2. Disruption of the p14/ARF-p53 pathways act as an independent negative prognostic factor in DLBCL.[29]

Inhibitory p21 $^{(Waf\ 1)}$ forms ternary complex with several CDK 2 complexes involving cyclin D, E and A. The promoter region of p21 has a p53 binding site. This confers wild type p53 inducibility initiating cell cycle arrest in G1. Thus p21 provides link between the response to DNA damage, induction of p53, inhibition of cell cycle and its displacement from cyclin-CDK complexes. p21 can also inhibit DNA replication directly by binding to PCNA. Thus p21 arrests damaged DNA replication in S phase. p21

expression has been studied in conjunction with p53. Immunohistochemically detected over-expression of p53 protein and absence of p21(waf1) expression correspond to loss of function of the P53-gene in diffuse large B-cell lymphoma (DLBCL) patients.[30]

p27 $^{(Kip\ 1)}$ suppresses CDK2-cyclin E activity. p27 plays a role in cellular differentiation, apoptosis and chemotherapeutic response. p27 has been reported to be indicative of a poor prognosis in malignant lymphoma.[31]

The p53 gene referred to as "the guardian of the genome" monitors DNA integrity by arresting cells at G1 to allow time for repair or programming them to cell death (apoptosis) when damage is too extensive and DNA is beyond repair. P53 abnormalities have been detected in about 15-20% of DLBCL[16] and found to be a prognostic indicator of clinical drug resistance and decreased survival.[32,33] Inactivation of p53 usually results from missense mutations in core region of the p53 gene coding for the sequence-specific DNA-binding domain. The resulting mutated p53 protein might gain new oncogenic properties.[34] Overexpression of p53 protein is relatively common in aggressive lymphomas. and has been found to be an independent factor in survival using multivariate analysis. Recent studies have shown that TP53 mutations in GC-DLBCL are associated with poor outcome. [35]

PR domain containing 1 with zinc finger domain (PRDM 1)/B lymphocyte – induced maturation protein 1 (BLIMP 1) is a transcriptional repressor expressed in a subset of germinal center (GC) B cells and in all plasma cells, and required for terminal B cell differentiation. BLIMP 1 is a tumour suppressor gene whose inactivation may contribute to lymphomagenesis by blocking post – GC differentiation of B cells toward plasma cells. The frequent inactivation of BLIMP1 specifically in ABC-DLBCL suggests an

important role for this gene in the pathogenesis of this lymphoma subtype. The loss of function of this gene contribute to the pathogenesis of ABC-DLBCL.[36]

MDM2 is a negative regulator of P53. p53 activity and levels are controlled by Mdm2 oncoprotein ubiquitin-dependent proteasomal degradation. During DNA damage, the interaction between p53 and Mdm2 is reduced, which allows p53 levels to accumulate. [37] Mdm2 protein binds p53 and exports it from the nucleus thus blocking its activity. Mdm2 also targets p53 for proteasome dependent degradation through its activity as a ubiquitin ligase. In the absence of P53, MDM2 can act as an oncogene, functionally suppressing P53 activity.

Imbalance of cell proliferation and death is a universal feature of malignancies including DLBCL. Tumour growth occurs as a result of imbalance between cell proliferation and cell death. Several studies have examined the impact of cell proliferation in DLBCL. High expression of Ki67, a protein detected in the G1,S,G2 and M phases of the cell cycle but not in G0, is a widely accepted proliferation marker. Proliferative index as measured by the Ki-67 fraction has shown to be an independent factor for survival. High Ki-67 expression is a poor prognostic indicator and may predict a reduced overall survival (OS) in DLBCL, independent of other clinical variables included in the IPI.[38,39]

The evolution of a normal cell into cancer involves disruption and deregulation of a number of basic cellular mechanisms. Apoptosis control mechanisms appear to be impaired in virtually all tumours suggesting that a required step in carcinogenesis is to disengage the apoptotic machinery. Bcl-2 family proteins play a key role in regulation of mitochondrial integrity and programmed cell death. It includes proteins with both antiapoptotic and proapoptotic function. Bcl-2 the first identified member of this family

prevents apoptosis as do Bcl-XL and Mcl-1. The antiapoptotic molecules Bcl-2 and Bcl-XL localize to the mitochondrial membrane and maintain mitochondrial integrity by allowing maintenance of respiration. Alternatively they bind and inhibit the function of proapoptotic family members. The multidomain proapoptotic molecules Bax and Bak are required for mitochondrial apoptotic pathway. Bax and Bak antagonize the function of the antiapoptotic family members and are critical in promoting apoptosis through the mitochondrial pathway. A third type of Bcl-2 family member – Bad, Bim and others termed BH3-only molecules (Bcl-2 homology domains) also play a key role in promoting apoptosis.

The bcl-2gene was originally discovered by virtue of its involvement in the translocation t(14;18) (q32;q21) in follicular lymphoma . This cytogenetic abnormality where it induces production of high levels of bcl-2 protein is also observed in about 20% of DLBCL. The bcl-2 protein located in the inner mitochondrial membrane functions as an antiapoptotic protein inhibiting cells from programmed cell death. Expression of bcl-2 protein in DLBCL has been described as an important adverse prognostic factor, independent of the IPI in multiple clinical trials. Bcl-2 protein expression predicts poor disease free survival (DFS) and overall survival (OS).[40] In a study where bcl-2 expression was assessed in the context of DLBCL subgroups,bcl-2 expression had a significant adverse effect on overall survival within the ABC subgroup. [41]

Bax is an important protein belonging to the bcl-2 family that functions as a death effector molecule which is neutralized by bcl-2. Bcl-2 disrupts formation of bax homodimers and operates as a death repressor. Bax expression delays the progression of

lymphoma by promoting apoptosis. Bax expression is a predictor of good OS and DFS in DLBCL.

Nuclear factor-kB (NF-kB) transcription factors play an important role in the regulation of immune and inflammatory response. [42] NF-kB is a small family of inducible transcription factors and exists in virtually all mammalian cells. Five NF-kB subunits, RelA (p65), RelB, c-Rel, p50 and p52, form various homodimers and heterodimers. In resting cell, NF-kB is sequestered and inactivated in the cytoplasm through interactions with its inhibitory proteins, which comprise IkBa, IkBb and IkBe, as well as the p105 and p100 precursors of p50 and p52, respectively. Upon stimulation by a variety of signals, inducible phosphorylation of IkB by the IkB kinase (IKK) accounts for its subsequent ubiquitination and proteasome-related degradation resulting in NF-kB nuclear translocation and transcriptional activation.[43] NF-kB signaling is generally considered to occur through either the canonical or alternative pathway.[44] In the canonical pathway, the NF-kB dimers (most commonly the p50/p65 dimers) are released and translocated to the nucleus with the activation of IKK complex (IKKa, IKKb and IKKg). In the alternative pathway, IKKa is independently activated for p100 processing to p52 leading to nuclear translocation of p52/RelB dimers. As a result of the activation of either pathway, NF-kB dimers could induce the expression of various genes regarding cell growth, differentiation, inflammatory responses and the regulation of apoptosis. Constitutive activation of the NF-kB has been documented to be involved in the pathogenesis of many human malignancies, including hemopoietic neoplasms. NF-kB has been shown to play an important role in a variety of cellular processes, including cell cycle regulation and apoptosis. Activation of the NF-kB pathway, with increased expression of pro-

proliferative and antiapoptotic genes is a characteristic of aggressive subtypes of DLBCL. Increased expression of NF-kB target genes are frequently seen in ABC-DLBCLs. [45] Conversely, GCB-DLBCL express low levels of NF-kB target genes.

Flow cytometry (FCM) plays multiple roles in the diagnosis, classification and prognostication of non-Hodgkin's lymphoma.[46] It has become an important tool in the diagnosis of mature lymphomas and the determination of prognosis in select cases.[47] Flow cytometry can be used to rapidly assess cell proliferation and DNA content (ploidy) within tumour from paraffin embedded tissues. These easily quantifiable biologic features such as cell proliferative activity and DNA ploidy may provide additional prognostic information beyond that derived from the clinically based IPI. Proliferative activity determined by FCM (%S-phase) predicts outcome in DLBCL.[48] FCM analysis of DNA content (ploidy) is a powerful guide to prognosis in many malignant diseases. Proliferative activity and aneuploidy correlates with morphological grading of tumours. High proliferative activity is associated with poor survival.

CHAPTER 4

SCOPE AND PLAN OF WORK

1. To profile patients with DLBCL based on their molecular characteristics and to study differences in behaviour of various groups.

2. To identify key molecule (if any) which has a pivotal role in driving tumour cell proliferation and survival. This candidate molecule can be further studied for targeted therapeutic intervention.

3. Identification of groups with poor prognostic outcome would help clinicians to target such groups with early, aggressive, multimodal therapy.

CHAPTER 5

MATERIALS AND METHODS

5.1 MATERIALS

One hundred and forty four consecutive cases of DLBCL (including live/dead patients) who had been treated at Cancer Institute (WIA), Chennai have been included in the study (**Table 1**). Patients who declined treatment or did not complete the prescribed treatment due to extraneous reasons (logistic or family related) were not included. However all patients who under treatment progressed or experienced toxicity necessitating change of regimens were included in the analysis.

Table 1. Patient Characteristics (n=144)

Patients Characteristics	No. (%) of Patients
Age (Yr)	
≤ 60	117 (81.3)
≥ 60	27 (18.8)
Stage	
1-2	65 (45.1)
3-4	79 (54.9)
Performance	
0-1	92 (63.9)
>2	52 (36.1)
LDH	
≤ 1 x normal	3 (2.1)
≥ 1 x normal	141 (97.9)
Extra nodal involvement	
≤ 1	127 (88.2)
≥ 1	17 (11.8)
IPI Score	
0-2	92 (63.8)
3-5	52 (36.2)

5.2 METHODS

5.2.1. IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on formalin-fixed, paraffin embedded sections. Five micrometer paraffin sections on APES coated glass microslides were used.The following steps were carried out.

1. Dewaxing and Rehydration: Sections were deparaffinized and rehydrated by consecutive submersions in Xylene (twice for 8 mins), absolute ethanol (twice for 3 mins) and hydrated in water (5 min).

2. Blocking of Endogenous Peroxidase action: Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide for 10 mins at room temperature and slides washed in Tris Buffer Saline (TBS pH 7.6).

3. Antigen retrieval: The sections were then subject to antigen retrieval in Tris EDTA buffer (TE buffer pH 9).

4. Incubation with specific primary antibody: After cooling down to room temperature, the sections were washed in TBS and incubated with the specific antibodies overnight at room temperature in a humid chamber. Following day, the slides were washed with TBS for 5 mins three times to remove the unbound primary antibody.

5. Incubation with Labelled Polymer (EnVision^{+} System-HRP) (DAKO): Tissue sections were then incubated at room temperature for 2 hours with Labelled Polymer. The slides were then washed with TBS for 5 mins three times to remove unbound polymer.

6. Incubation with substrate-chromogen: The sections were then incubated with liquid DAB+Substrate-chromogen for 5-10 mins.

7. Counterstaining and mounting: The sections were then counterstained with haematoxylin, dehydrated, cleared and mounted using DPX.

Negative control slides were processed under same conditions as above omitting the primary antibody.

Table 2 gives an overview of the details of antibodies used, their source, dilution and cut off points for analysis.

Table 2 : List of antibodies used in the study

Protein	Clone	Source	Dilution	Cut-off (%)
CD10	56C6	Leica	1:10	>10 positive cells
Bcl-6	PG-B6p	DAKO	1:20	>10 positive cells
Mum	MUM1p	DAKO	1:50	>80 positive cells
Blimp	6D3	Santa-Cruz	1:50	>30 positive cells
Cyclin D2	M-20	Santa-Cruz	1:50	>20 positive cells
Cyclin E	13A3	Leica	1:10	>10 positive cells
Cyclin A	6E6	Leica	1:100	>10 positive cells
Cyclin B1	7A9	Leica	1:20	>50 positive cells
p16	JC-8	Santa-Cruz	1:50	>10 positive cells
p14/ARF	4C6/4	Cell Signalling Technology	1:100	>40 positive cells
p21	187	Santa-Cruz	1:50	>10 positive cells
p27	1B4	Leica	1:20	>10 positive cells
p53	DO-7	DAKO	1:50	>10 positive cells
Mdm2	1B10	Leica	1:50	>10 positive cells
Bcl-2	124	DAKO	1:100	>50 positive cells
Bax	Rabbit anti-human	DAKO	1:200	>10 positive cells
NFkB/p65	F-6	Santa-Cruz	1:50	Nuclear stain
Ki-67	MIB-1	DAKO	1:150	>80 positive cells

5.2.2. FLOWCYTOMETRY STUDY

Flowcytometric analysis was done on archival specimens of sample group of study patients. The procedure consisted of taking five to six 50µ thick sections from representative paraffin block. After dewaxing and rehydration, the tissue was minced and digested in 2ml of 0.5% pepsin solution (pH 1.5). The contents were then filtered

through cotton gauze to get as many cells as possible. The samples were rinsed once in 1-2ml PBS (Phosphate buffered saline), pH7.4 and re-suspended in 1ml PBS. Cell concentration was adjusted to 5.0 x 10^5 cells / ml. The sample was then centrifuged at 400g for 5 minutes.

Supernatant was removed and to the pellet, 250µl of solution A (Trypsin buffer) was added, mixed gently and incubated for 10 minutes at room temperature. Then 200µl of solution B (Trypsin Inhibitor + Ribonuclease A) was added and contents incubated at room temperature. Next 200µl of cold solution C (Propidium iodide) was added and incubated in dark for 10 minutes at room temperature. The sample was analyzed in flowcytometer for nuclear DNA.

5.2.3. P53 MUTATION ANALYSIS

5.2.3.1 MATERIALS

Lymph node biopsy samples were collected from patients after getting the informed consent. Fresh tissue samples of 40 patients that was available (out of 144 patients) were included in this study. The tissue samples were collected and stored immediately in liquid nitrogen and then DNA extraction was performed.

5.2.3.2 DNA EXTRACTION

DNA was extracted from tissues using QIAamp DNA tissue Kit according to the manufacturer's instructions.

5.2.3.3 ANALYSIS OF THE QUALITY AND QUANTITY OF THE DNA

The extracted DNA was run on a 0.8% agarose gel and stained with ethidium bromide to check for the quality of the DNA. The gel was visualized using Imagemaster Totallab gel documentation system (Amersham).

One micro liter of the DNA sample was quantified using Nanodrop ND_1000 spectrophotometer. The ratio of absorbance at 260/280 nm was used to assess the purity of DNA.

5.2.3.4 PCR FOR TP53 GENE

PCR for exon 2-11 of the TP53 gene was done in a 25µl reaction containing 10mMTris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 100µM dNTP's each, 0.4µM of each primer, and 1U of Taqpolymerase (Hot start Taq polymerase, Medox). The primer sequences used to amplify exons 5-8 were as published by Vet et al (1994), and for exon 2-4 and 9-11 were as published by Verselis et al (2000). The PCR cycling steps comprised initial denaturation at 95°C for 10 minutes, subsequent 35 cycles included denaturation at 94°C for 20s, annealing at 60°C for 20s (exons 5-8) and a touchdown protocol of 65-55/55°C for exons 2-4 and 9-11 for 20s and extension at 72°C for 45s and a final extension at 72°C for 10 min.

5.2.3.5 GEL EXTRACTION

The specified PCR bands were cut from the agarose gel with a clear sharp scalpel or sterile blade. Gel extraction was carried out as per manufacturers protocol (Qiagen, gel extraction kit). Briefly, three volumes of QG buffer was added to the gel slice and incubated at 50^oC for 10min. After the gel slice was completely dissolved one volume of isoproponal was added, mixed and spun at 12,000xg for one minute at room temperature. The flow through was discarded and 0.5ml of buffer QG was added and spun for one minute. Wash buffer (0.75mL of PE) was added and washings were done. Finally DNA was eluted with10-15µl of elution buffer.

5.2.3.6 DNA SEQUENCING

Sequencing was performed bi-directionally using Big Dye terminator kit v3.1 (Applied Biosystem, Foster City, CA) according to manufacturer's instructions. The PCR conditions for the cycle sequencing are: Initial denaturation at 96^{o}C for 10 min, denaturation at 94^{o}C for 10sec, annealing at 50^{o}C for 5sec, extension at 60^{o}C for 4mins for a total of 30 cycles, using only forward primer. Cycle sequencing reaction was set up as follows (**Table 3**):

Table 3: Reaction set up for cycle sequencing

	Sequencing Reaction Dilutions			
Reaction Constituents	**Full reaction (>1000bp)**	**Half reaction (>550bp)**	**One-Fourth (>300-550bp)**	**One-Eighth (<300 bp)**
Ready reaction mix (2.5 X)/ ul	8	4	2	1
Dilution Buffer (5X)/ul	-	-	1	1.5
Template (200 ng/ul)	X	X	X	X
Primer (0.8 pmol/ul)	4	2	2	2
MQ water	Y	Y	Y	Y
TOTAL(ul)	20	10	10	10

5.2.3.7 CLEAN-UP PROCEDURE FOR 96 WELL PLATE

To a 10 ul reaction in a 96-well plate 10 ul of MilliQ water and 2 ul of 125mM EDTA was added. To this 2 ul of 3M sodium acetate pH 4.6 and 50 ul of 100% ethanol was added to remove excess dNTPs, RR mix and unincorporated primers. The contents were mixed well, sealed and incubated at room temperature for 15 minutes and centrifuged at

a speed of 3000g for 30 minutes in room temperature. The supernatant was decanted by inverting the plate on paper towel and spinned upto 180g to remove residue supernatant. 100 ul of 70% ethanol was added, sealed and spinned at 3000g for 5 minutes. The supernatant was decanted and again 70% ethanol wash was repeated. The pellet was dried at room temperature and resuspended in 10µl of Hi-dye formamide, denatured at 95^{o}C for 5min, snap freezed and then loaded into ABI 3500DX Genetic analyzer (Applied Biosystems by Life technologies, Foster City, CA).

CHAPTER 6

RESULTS AND ANALYSIS

6.1 IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on all the 144 samples and their results with OS are listed in **Table 4**.

Table 4: Univariate analysis for overall survival in DLBCL patients (n=144)

Factor	No. of cases (%)	Overall survival (%)					p-value
		1 yr	2 yr	3 yr	4 yr	5 yr	
IPI							**0.000***
0-2	92 (63.8)	91.3	80.4	76.6	73.9	70.2	
3-5	52 (36.2)	76.2	49.4	42.3	39.1	32.6	
Immunophenotype							**0.013***
GCB	70 (48.6)	88.5	78.3	73.1	71.2	68.6	
ABC	74 (51.4)	82.1	61.1	56.7	53.0	47.2	
CD10							0.089
Positive	57 (39.6)	89.4	75	70.7	68.1	68.1	
Negative	87 (60.4)	83.7	65.9	60.8	57.8	51	
Bcl-6							0.175
Positive	53 (36.8)	83	73.5	71	71	66.3	
Negative	91 (63.2)	87.7	67.1	60.8	56.5	52.9	
Mum							0.608
Positive	17 (11.8)	76.5	64.7	58.2	58.2	58.2	
Negative	127 (88.2)	87.2	70.2	65.6	62.5	58.2	
Blimp							0.106
Positive	5 (3.5)	80	60	40	20	20	
Negative	139 (96.5)	86.1	69.9	64.8	62.8	60	
Cyclin D2							0.425
Positive	22 (15.3)	81.8	72.7	67.5	67.5	67.5	
Negative	122 (84.7)	86.7	69.0	64.2	60.8	56.0	
Cyclin E							0.28
Positive	56 (38.9)	82.0	60.1	56.2	56.2	56.2	
Negative	88 (61.1)	88.4	75.6	70.1	65.6	59.1	
Cyclin A							0.381
Positive	140 (97.2)	85.5	68.7	63.6	60.7	56.4	
Negative	4 (2.8)	100	100	100	100	100	
Cyclin B1							0.575

	Positive	15 (10.4)	86.7	60	60	60	60	
	Negative	129 (89.6)	85.9	70.7	65.4	62.4	58.3	
p16								0.292
	Positive	50 (34.7)	87.5	70.4	70.4	70.4	65.7	
	Negative	94 (65.3)	85.1	69.1	64.6	58.1	54.3	
p14								0.325
	Positive	71 (49.3)	78.5	62.5	60.9	58.7	58.7	
	Negative	73 (50.7)	93.1	80.6	68.7	65.4	58.8	
p21								**0.026***
	Positive	37 (25.7)	89.0	83.4	74.8	74.8	74.8	
	Negative	107 (74.3)	84.9	64.8	61.3	57.1	51.2	
p27								0.468
	Positive	127 (88.2)	84.9	68	63.5	60.5	57.5	
	Negative	17 (11.8)	94.1	81.1	73.0	73.0	58.4	
p53								0.899
	Positive	107 (74.3)	86.9	68.9	64.4	61.8	59.7	
	Negative	37 (25.7)	83.0	77.3	65.0	61.7	53.9	
Mdm2								0.103
	Positive	138 (95.8)	86.8	71.2	66.0	63.1	58.9	
	Negative	6 (4.2)	66.7	33.3	33.3	33.3	33.3	
Bcl-2								0.065
	Positive	87 (60.4)	83.8	62.8	58.7	58.7	53.0	
	Negative	57 (39.6)	89.2	80.1	74.0	71.7	65.4	
Bax								0.281
	Positive	66 (45.8)	83.2	67.4	57.6	55.3	52.6	
	Negative	78 (54.2)	88.3	71.4	70	69.9	61.7	
NFkB								**0.018***
	Positive	96(66.6)	85.2	63.8	59.2	56.4	50	
	Negative	48 (33.4)	87.4	81	75.7	72.9	72.9	
Ki-67								0.936
	Positive	81 (56.3)	83.7	67.2	63.8	62.0	59.1	
	Negative	63 (43.7)	88.8	77.5	65.6	61.7	56.8	

6.2 FLOWCYTOMETRY

FACS canto II (Becton Dickinson) flowcytometer equipped with DDM (doublet discrimination module) and software FACS Diva were utilized for the study after quality assurance for the equipment and DNA study using appropriate quality control reagents.

DNA Index and S-phase fraction were subjected to multivariate analysis. Out of the 144 cases studied for immunohistochemical expression of various proteins, only 55 paraffin blocks had sufficient materials to carry out flowcytometric analysis of cell cycle and ploidy. The S-phase fraction was increased (>10%) in 33 cases and decreased (<10%) in 22 cases. The tumours were diploid in 9 cases and hypoploid in 8 cases and hyperploids in 38 cases. The data analysis was done by using Sequencing Analysis v3.2.1 software.

6.3 PCR FOR TP53 GENE

All the coding exons of *TP53* gene, i.e. exons 2-11 were amplified by PCR and run on 1.2% agarose gel.

6.4 SEQUENCING OF *TP53* GENE

Sequencing for exon 2-11 was done for all the forty samples. The PCR products were directly sequenced with forward primers first and then with reverse primers for the samples which showed a change in a particular exon. The mutations and variations were designated according to HUGO recommendations, with nucleotide number starting at the first transcribed base of *TP53* gene according to GenBank entries NM_000546.4, UniProt_P04637 respectively.

6.5 STATISTICAL ANALYSIS

Descriptive statistics was done using one way frequency table. Kaplan Meier method was employed to estimate OS. Log rank test was used to test for differences between survival

curves. Cox proportional hazard model was used for multifactorial analysis of prognostic factors for OS. All the above were performed using SPSS software version 17.0.

CHAPTER 7

DISCUSSION

7.1 IMMUNOHISTOCHEMISTRY

This study identified p21 and NFkB as the only two statistically significant variables which can be used to predict prognosis in DLBCL. Expression of NFkB in ABC phenotype identified patient group with the worst 5 years OS of 39.6%. Similarly loss of p21 expression in ABC phenotype identified the patient group with worst 5 years OS of 37.2%. ($p = 0.029$)

The frequency of Bcl-2 positivity in this study was 87 (60.4%) **(Figure 2)** and Bcl-2 negativity was 57 (39.6%). Conventionally Bcl-2 positivity has been associated with chemoresistance and poor OS. This study has shown a trend supporting the association of Bcl-2 positivity with overall poor survival **(Figure 3)**. The difference in OS between Bcl-2 positive and Bcl-2 negative was more apparent from the 2^{nd} year of follow-up and continued up to 5 years when analyzed by Kaplan Meier method. This difference reached close to statistical significance ($p = 0.065$), however, failed to reach statistically significant value ($p = <0.05$). An increase in the number of patients and further follow-up could establish the discriminative value of this protein by gaining statistical significance.

Figure 2: Immunohistochemical expression pattern o f Bcl-2

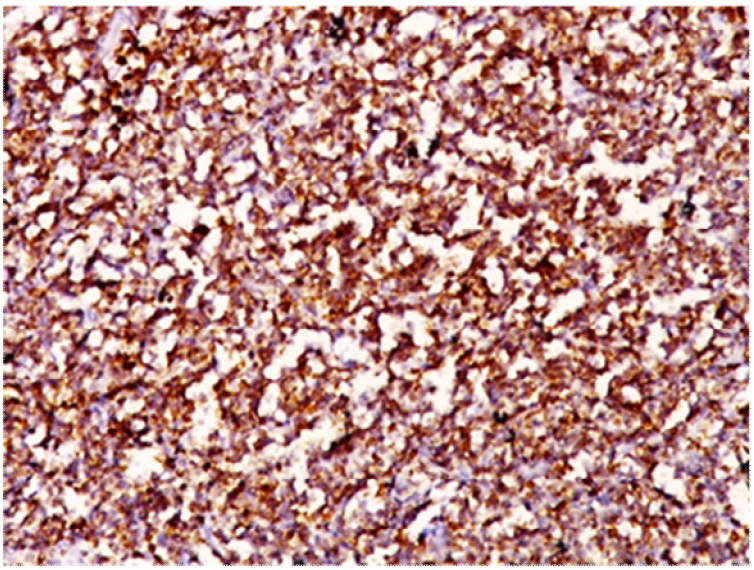
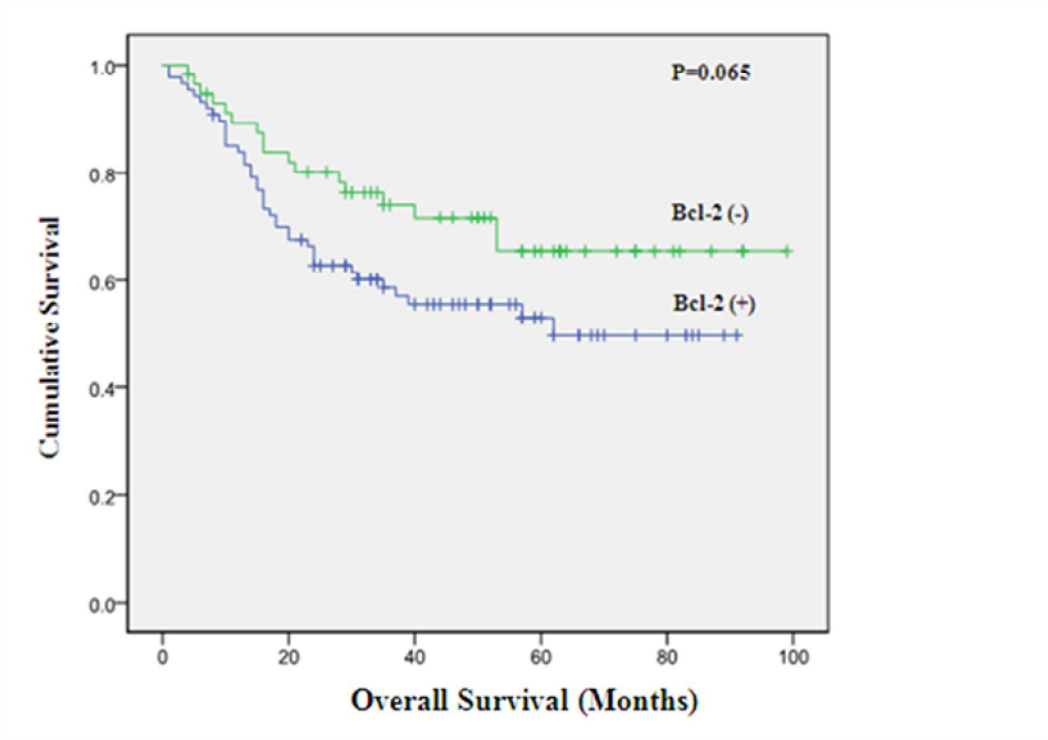


Figure 3: Kaplan-Meier Overall survival curve for expression of Bcl-2



The frequency of Bax positivity in this study was 66 (45.8%) **(Figure 4)** and Bax negativity was 78 (54.2%). Traditionally Bax positivity has been associated with

increased OS. Paradoxically this study has shown a slight trend of Bax positivity being associated with poor OS **(Figure 5)**. This observation was not statistically significant (p = 0.281). However, several combinations of Bax/Bcl-2 protein expression were found to have significantly different OS. Four subgroups of Bax/Bcl-2 protein expression were created namely subgroup 1(Bax+/Bcl-2+), subgroup 2 (Bax+/Bcl-2-), subgroup 3 (Bax-/Bcl-2+) and subgroup 4 (Bax-/Bcl-2-) and their OS studied (**Table 5**). Interestingly, it was noted that subgroup 4 (Bax-/Bcl-2-) comprising of 28 patients had a favourable 5 years survival rate of 79.4% compared to the other combination which had an average 5 years survival rate of around 50% **(Figure 6)**. This was statistically significant: subgroup 1 vs 4 (p = 0.018), 2 vs 4 (p = 0.022), 3 vs 4 (p = 0.008). This finding is intriguing and warrants further study to elucidate the interplay between Bax/Bcl-2 in regulating apoptosis and its effect on OS and response to therapy in DLBCL.

Figure 4: Immunohistochemical expression pattern of Bax

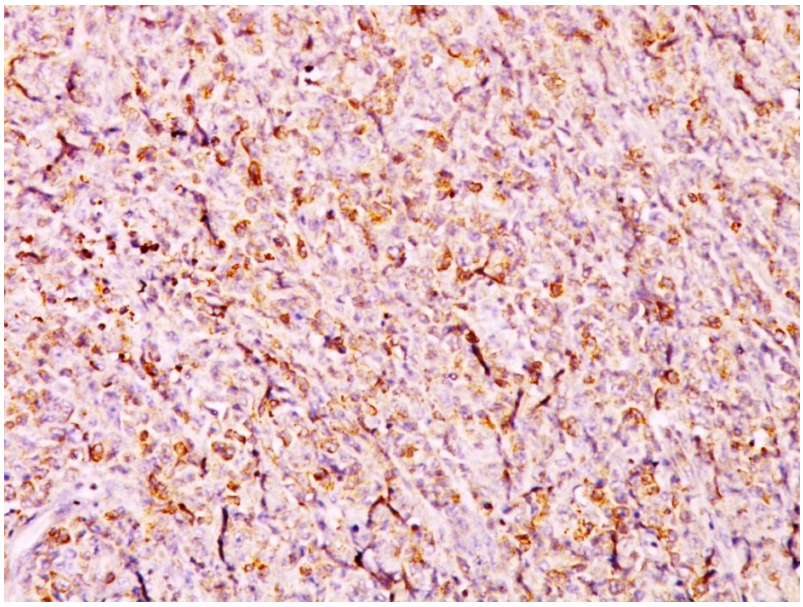


Figure 5: Kaplan-Meier Overall survival curve for expression of Bax

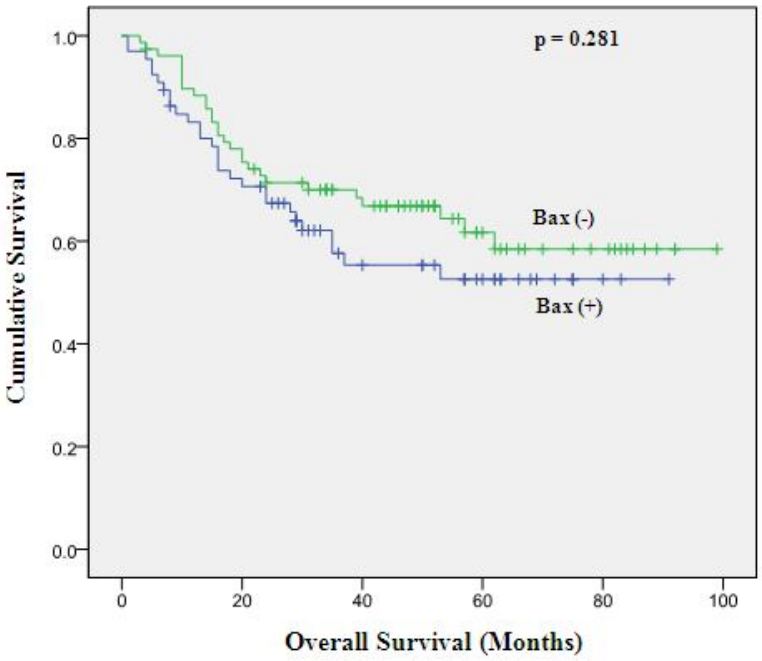


Figure 6: Overall survival of combination of Bax/Bcl-2 expression

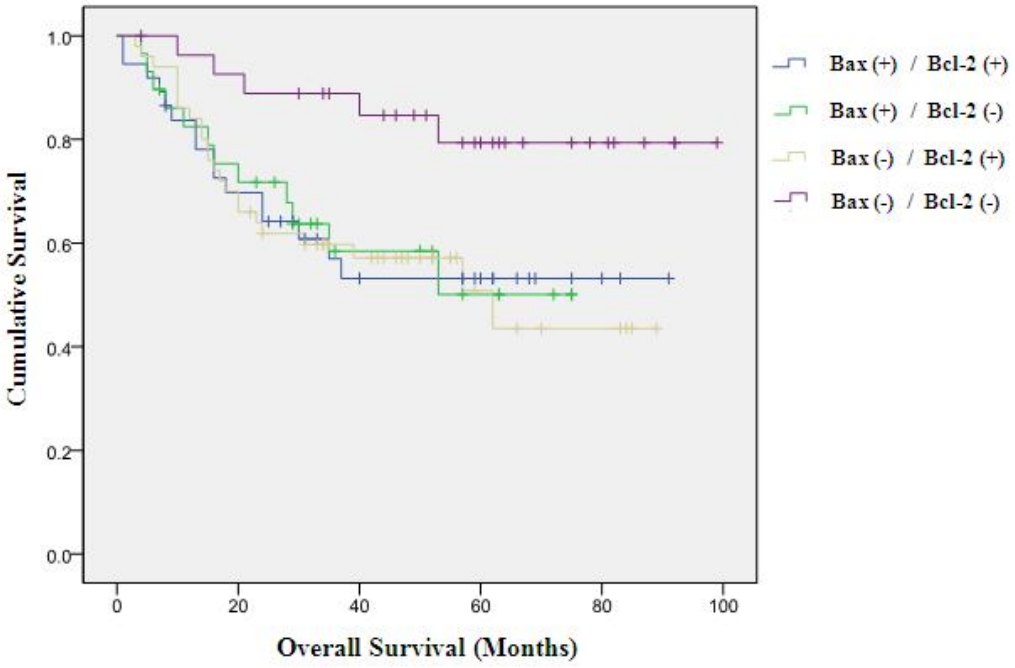


Table 5: Overall Survival of combination of Bcl-2 / Bax expression

Combination	Subgroup	Overall survival (%) 1 yr	2 yr	3 yr	4 yr	5 yr	p-value
Bax+ / Bcl-2+	1 (37)	83.7	64.2	57.0	53.2	53.2	0.070
Bax+ / Bcl-2-	2 (29)	82.5	71.7	58.4	58.4	50.1	
Bax- / Bcl-2+	3 (50)	84.0	61.9	59.7	57.1	50.8	
Bax- / Bcl-2-	4 (28)	96.3	88.9	88.9	84.7	79.4	

Note: 1 Vs 4 (p = 0.018), 2 Vs 4 (p = 0.022),
3 Vs 4 (p = 0.008)

NFkB was positive in 96 patients (66.6%) **(Figure7)** and negative in 48 patients (33.4%). NFkB positivity has been associated with poor response to treatment and poor OS. This study showed that NFkB positivity was associated with a 5 years survival of only 50% whereas, NFkB negativity was associated with a survival of 72.9% and this finding was statistically significant (p = 0.018) **(Figure 8)**. To further increase the discriminating value of NFkB and to identify subgroups which can be targeted for therapeutic intervention, four subgroups based on NFkB expression and DLBCL immunophenotype (GCB,ABC) were created namely subgroup 1 (NFkB+/GCB), subgroup 2 (NFkB-/GCB), subgroup 3 (NFkB+/ABC) and subgroup 4 (NFkB-/ABC) **(Table 6)**.

Subgroup 3 (NFkB+/ABC) was associated with a remarkably low 5 years OS of 35.6% when compared to the other 3 subgroups which had higher survival (subgroup 1 = 66.7%, subgroup 2 = 70.7%, subgroup 4 = 78.6%) and this was statistically significant - subgroup 1 vs 3 (p = 0.022), 2 vs 3 (p = 0.008), 4 vs 3 (p = 0.032) **(Figure 9)**. The association of subgroup 3 (NFkB+/ABC) has established the role of constitutive NFkB activation as a pathogenetic mechanism in ABC DLBCL conferring it with poor prognosis. Identification of this subgroup as a poor prognostic group and targetting them

with therapeutic interventions aimed at NFkB could yield favourable outcomes. Studies are underway to see whether identifying this subgroup as a separate entity and treated aggressively with NFkB specific blockage. [50]

Figure 7: Immunohistochemical expression pattern of NFkB

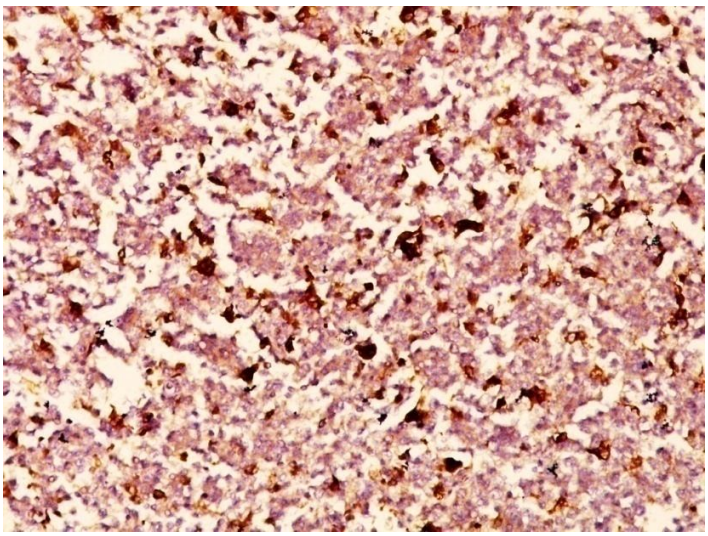


Figure 8 : Kaplan-Meier Overall survival curve for expression of NFkB

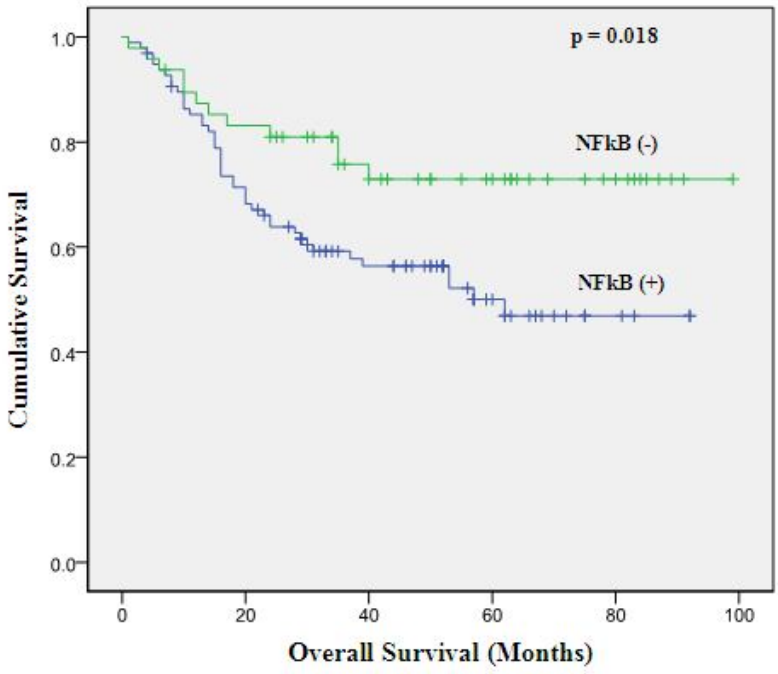
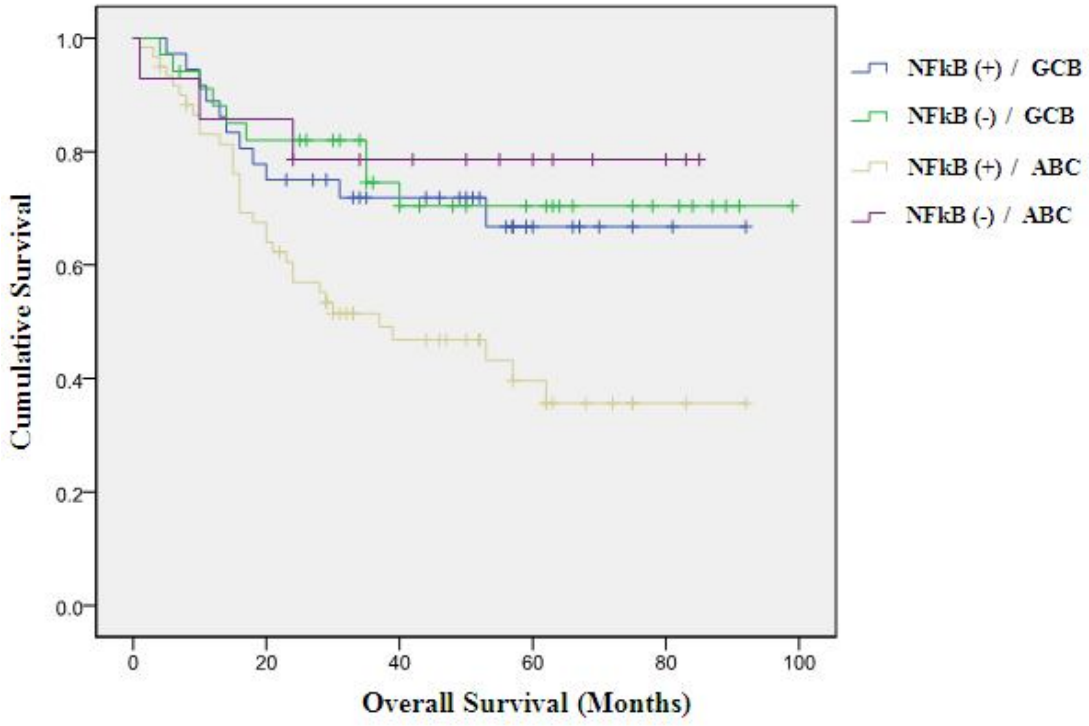


Table 6: Overall Survival of combination of NFkB expression / Immunophenotype

Combination	Subgroup (No. of cases)	Overall survival (%)					p-value
		1 yr	2 yr	3 yr	4 yr	5 yr	
NFkB+ / GCB	1 (36)	88.9	75.0	71.9	71.9	66.7	0.007
NFkB- / GCB	2 (34)	88.0	82.0	74.5	70.4	70.4	
NFkB+ / ABC	3 (60)	83.0	56.9	51.5	46.8	39.6	
NFkB- / ABC	4 (14)	85.7	78.6	78.6	78.6	78.6	

Note: 1 Vs 3 (p = 0.022), 2 Vs 3 (p = 0.008), 4 Vs 3 (p = 0.032)

Figure 9: Overall survival of combination of NFkB / Immunophenotype



Cyclin D2 was positive in 22 cases (15.3%) **(Figure 10)** and negative in 122 cases (84.7%). Few large studies[22] have found a correlation between Cyclin D2 positivity and inferior survival. This study showed a 5 year survival of 67.5% in Cyclin D2 positive

cases and a survival of 56% in Cyclin D2 negative cases. This was not statistically significant **(Figure 11)**.

Figure 10: Immunohistochemical expression pattern of Cyclin D2

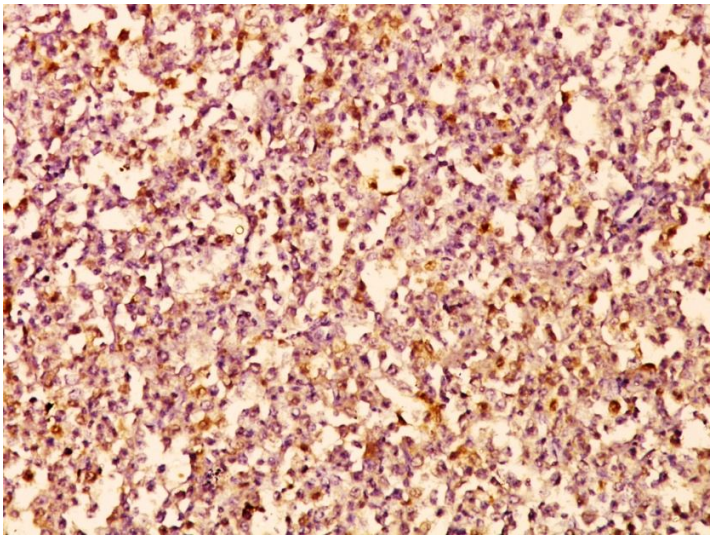
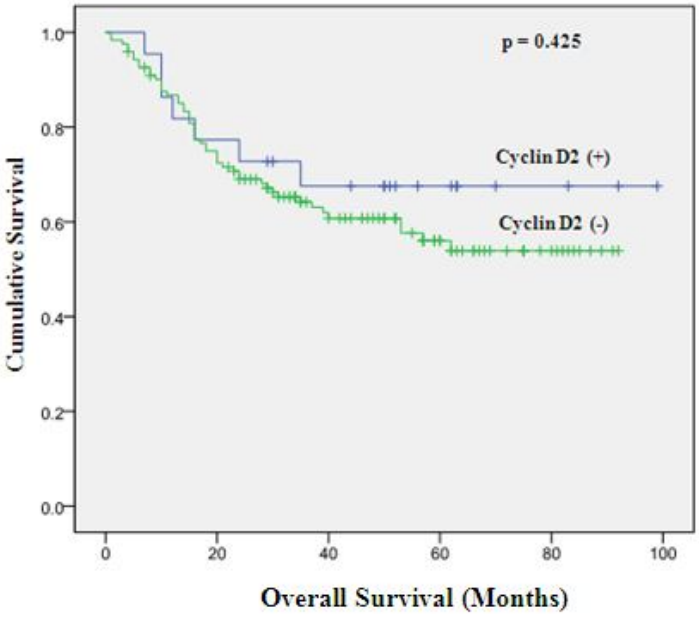


Figure 11: Kaplan-Meier Overall survival curve for expression of Cyclin D2



p16 was positive in 50 cases (34.7%) **(Figure 12)** and negative in 94 cases (65.3%). Loss of p16 expression is known to be associated with aggressive tumours and poor OS.[21, 28] This study also showed a trend of inferior 5 years survival (54.3%) in p16 negative cases compared to p16 positive cases (65.7%). However, this finding was not found to be statistically significant **(Figure 13)**. To further elucidate the interplay between p16 and Cyclin D2 on the modulation of the mitogenic effect of CDK4/6, four subgroups were created based on various combinations of p16 and Cyclin D2 expression namely subgroup 1 (p16+/Cyclin D2+), subgroup 2 (p16+/Cyclin D2-), subgroup 3 (p16-/Cyclin D2+) and subgroup 4 (p16-/Cyclin D2-) **(Table 7)**. Subgroup 2 (p16-/Cyclin D2+) having maximum mitogenic effect by Cyclin D2 positivity and loss of p16 expression was associated with aggressive tumour behaviour and lowest 5 years OS (52.7%). However, this observation did not reach statistical significance (p = 0.555).

Figure 12: Immunohistochemical expression pattern of p16

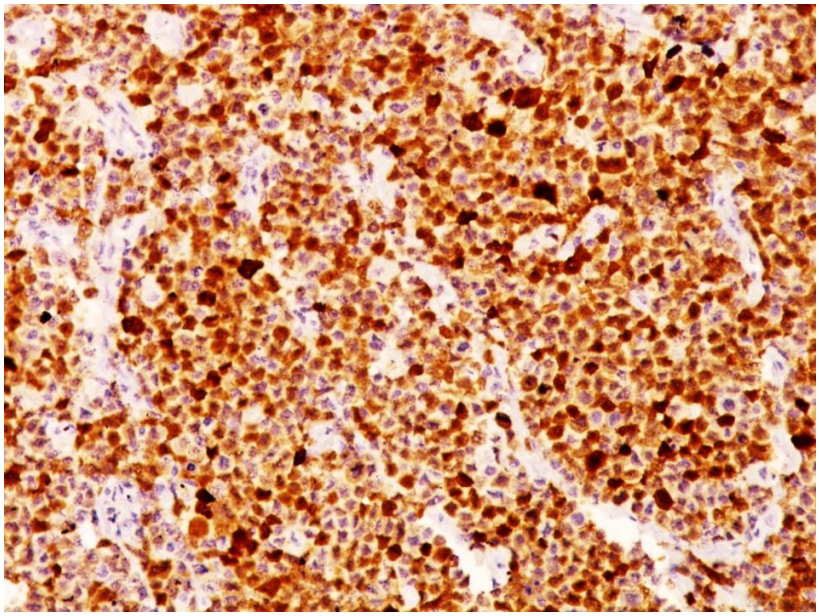


Figure 13 : Kaplan-Meier Overall survival curve for expression of p16

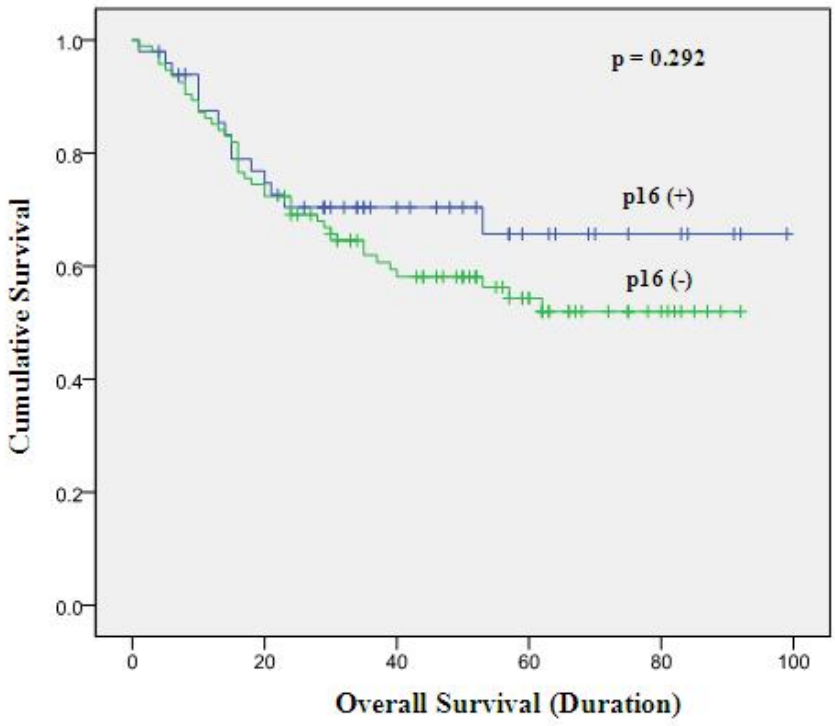


Table 7: Overall Survival of combination of Cyclin D2 / p16 expression

Combination	Number of Cases	Overall survival (%)					p-value
		1 yr	2 yr	3 yr	4 yr	5 yr	
p16+ / Cyclin D2+	9	88.9	88.9	88.9	88.9	88.9	0.555
p16- / Cyclin D2+	13	76.9	61.5	52.7	52.7	52.7	
p16+ / Cyclin D2-	41	87.3	66.0	66.0	66.0	59.4	
p16- / Cyclin D2-	81	86.4	70.3	63.5	59.0	54.8	

Cyclin E was positive in 22 cases (15.3%) **(Figure 14)** and negative in 122 cases (84.7%). This study showed an early survival benefit in Cyclin E negative cases, but overall 5 years survival was comparable **(Figure 15)**. This finding was not statistically significant (p = 0.28). In a study of 101 cases [24] overexpression of Cyclin E was associated with poor treatment response and inferior short term prognosis. However, this

study did not show any long term decrease in survival of Cyclin E overexpression cases compared to Cyclin E underexpression cases.

Figure 14: Immunohistochemical expression pattern of Cyclin E

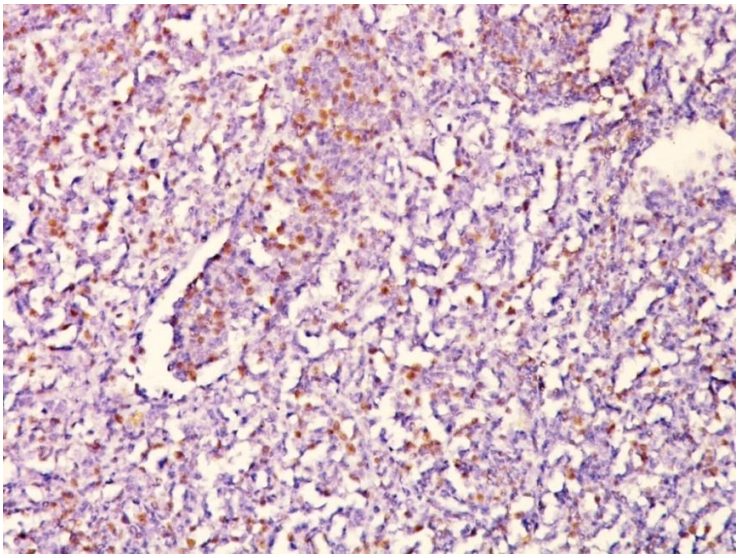
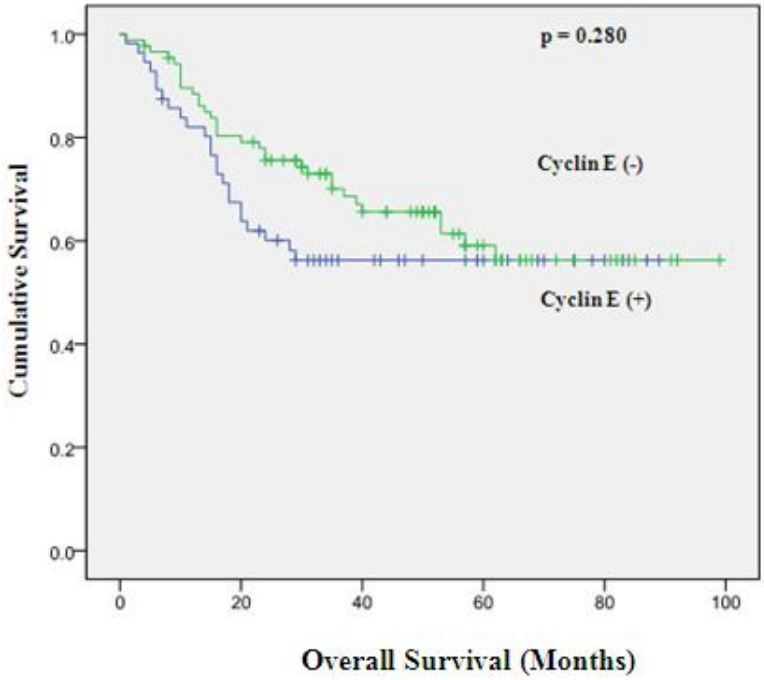


Figure 15 : Kaplan-Meier Overall survival curve for expression of Cyclin E



p27 was positive in 127 cases (88.2%) **(Figure 16)** and negative in 17 cases (11.8%) and did not show any statistical significance in OS **(Figure 17)**. In a large study of 671 patients[50] worst OS was found among patients with low p27 expression. To ascertain the interplay between Cyclin E and p27 in the modulation of the effect of CDK2 at the G1/S boundary 4 subgroups based on various combinations of p27 and Cyclin E expression **(Table 8)**. This comparison did not have any discriminative value and it did not reach statistical significance.

Figure 16 : Immunohistochemical expression pattern of p27

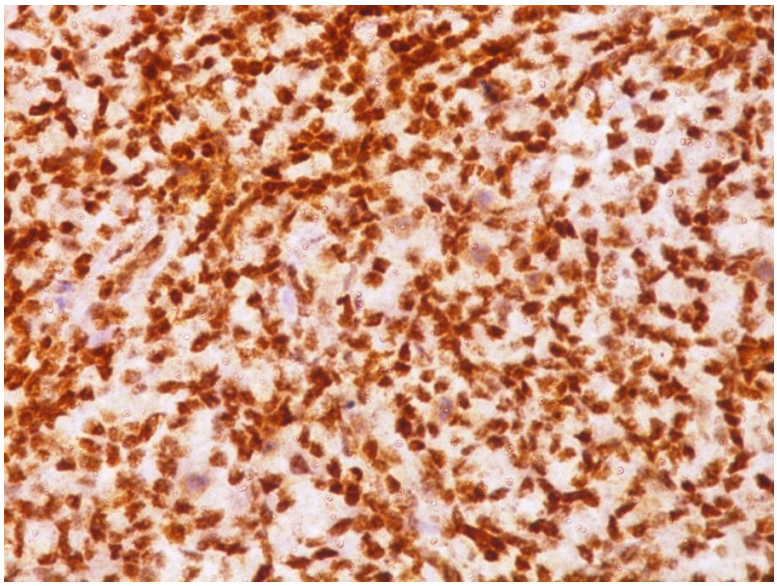
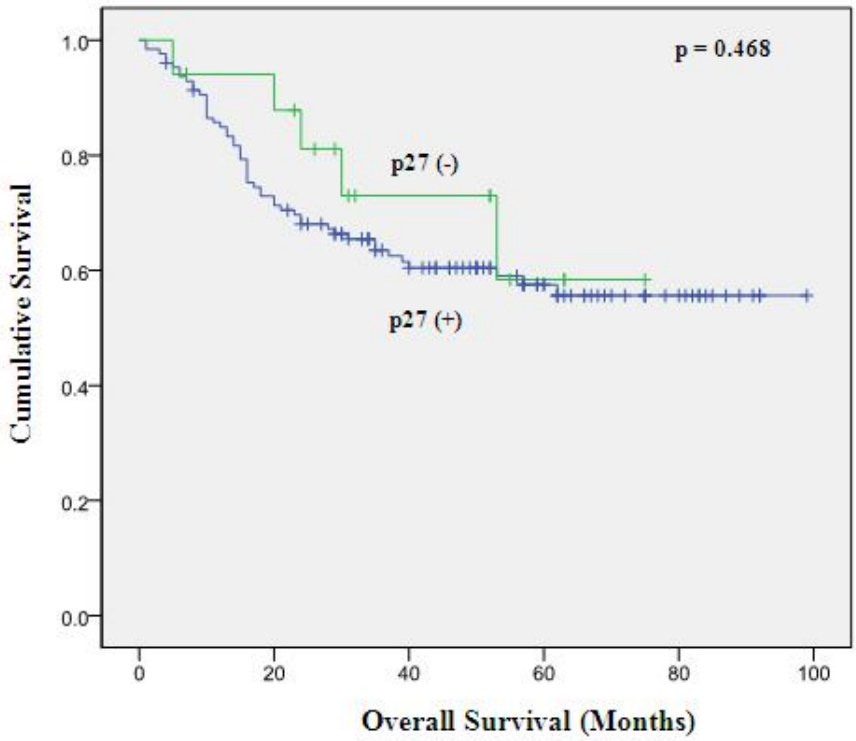


Table 8: Overall Survival of combination of p27 / cyclin E expression

Combination	Number of Cases	Overall survival (%)					p-value
		1 yr	2 yr	3 yr	4 yr	5 yr	
Cyclin E+ / p27 +	49	81.6	59.2	55.1	55.1	55.1	0.452
Cyclin E- / p27 +	78	86.9	73.7	68.9	63.9	59.1	
Cyclin E+ / p27 -	7	85.7	68.6	68.6	68.6	68.6	
Cyclin E- / p27 -	10	90.0	90.0	78.8	78.8	59.1	

Figure 17: Kaplan-Meier Overall survival curve for expression of p27



Cyclin A is associated with poor prognosis and survival. In this study 140 cases (97.2%) were positive for Cyclin A **(Figure 18)** and 4 cases (2.8%) were negative. Cyclin A negativity was associated with 100% survival at the end of 5 years compared to only 56.4% survival of Cyclin A negative cases **(Figure 19)**. Since only four patients were Cyclin A negative, a statistically significant conclusion could not be drawn from the above finding. Larger study would probably provide statistically significant results.

Figure 18: Immunohistochemical expression pattern of Cyclin A

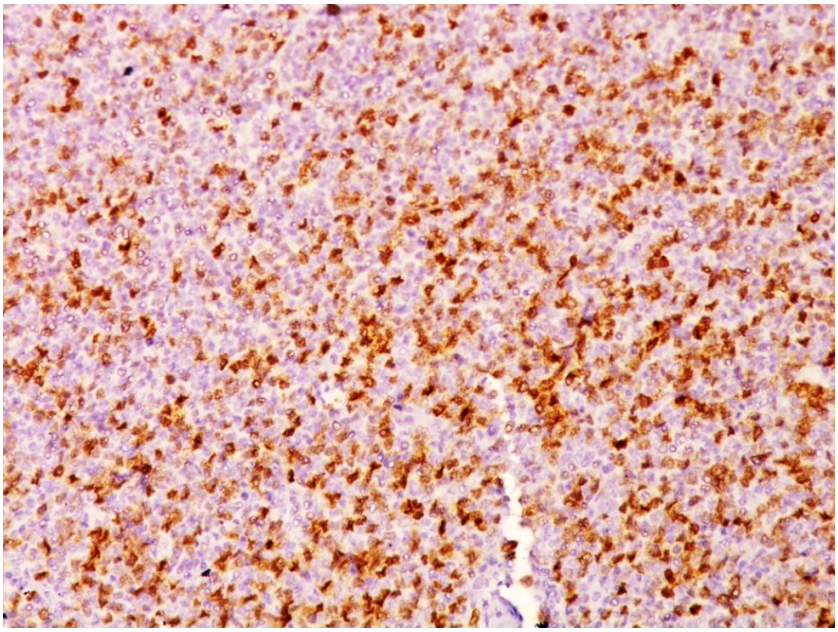
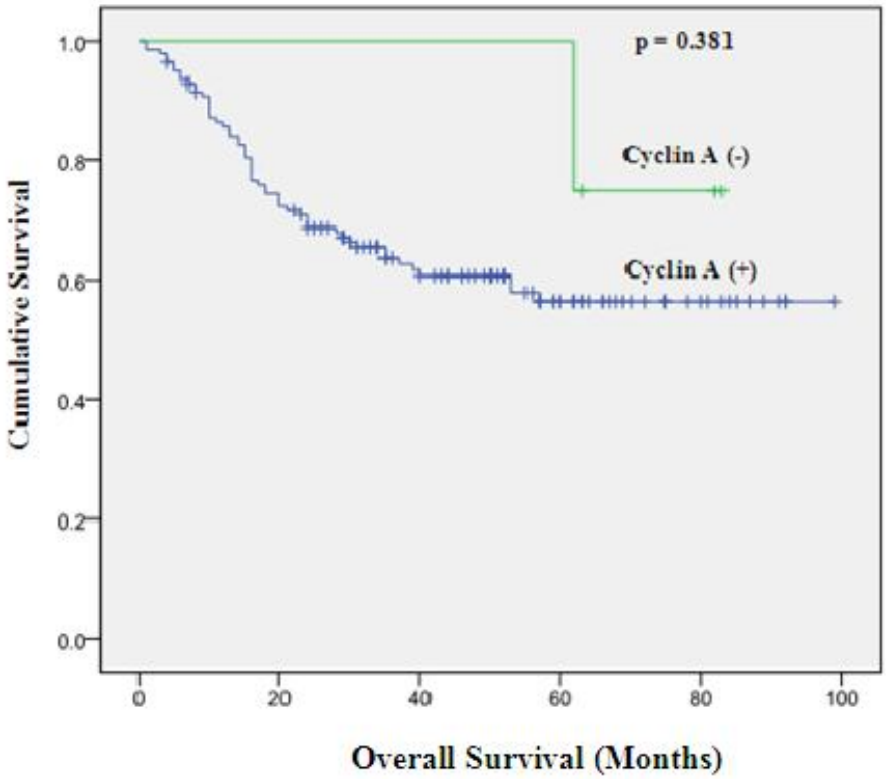


Figure 19:Kaplan-Meier Overall survival for expression of Cyclin A



Cyclin B was positive in 15 cases (10.1%) **(Figure 20)** and negative in 129 cases (89.6%). However no statistically significant difference was seen between the two groups **(Figure 21)**.

Figure 20: Immunohistochemical expression pattern of Cyclin B1

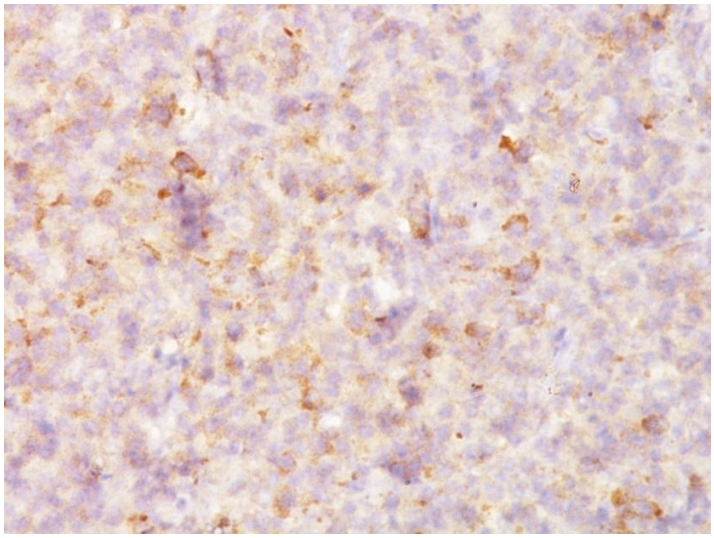
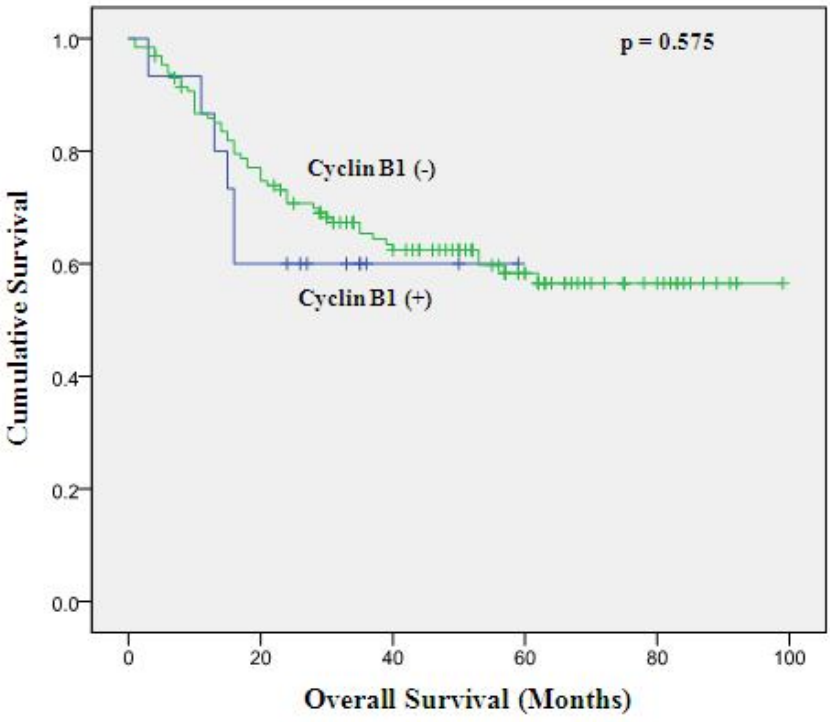


Figure 21 : Kaplan-Meier Overall survival curve for expression of Cyclin B1



p21 was positive in 37 cases (25.7%) **(Figure 22)** and negative in 107 cases (74.3%). Patients with p21 positive expression had statistically significant better overall 5 years survival (74.8%) compared to p21 negative cases (51.2%) (p = 0.026) **(Figure 23)**. A recent study has shown that the 5 year survival of p21 positive patients was statistically superior to p21 negative patients.

Figure 22: Immunohistochemical expression pattern of p21

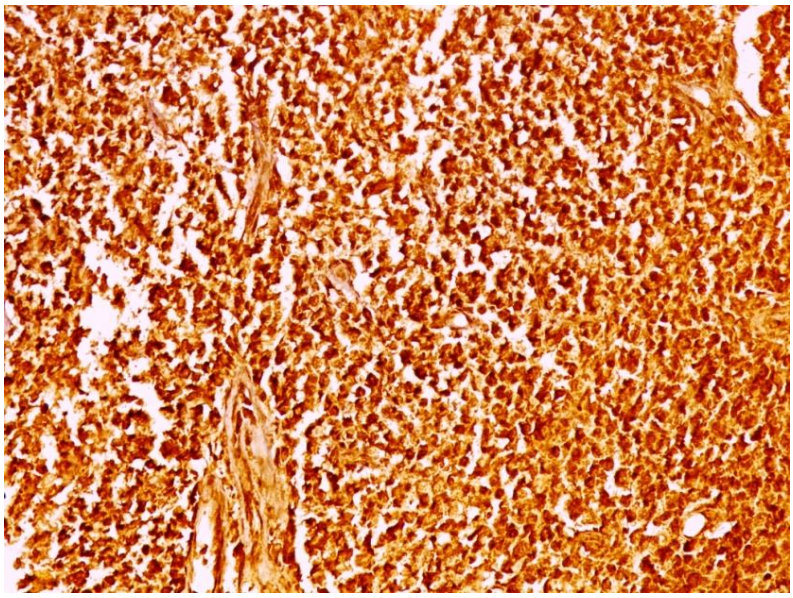
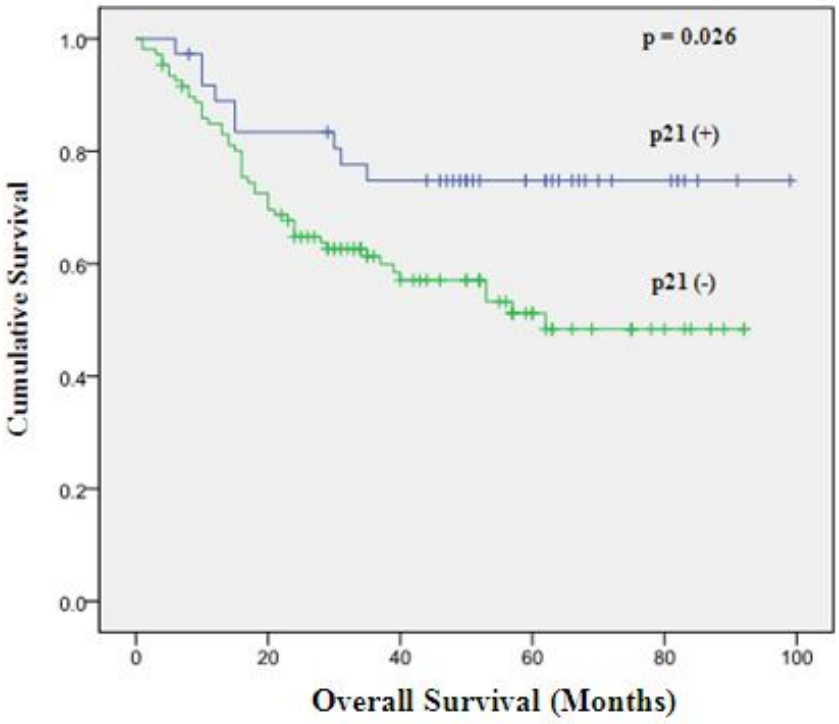


Figure 23: Kaplan-Meier Overall survival curve for expression of p21



Studies have suggested prognostic value of loss of p53 function associated with absence of p21 in GCB phenotype of DLBCL. This study did not show any significant influence of p21 expression on the outcome of patients with GCB phenotype. This may be due to other apoptotic pathways other than Bcl6 in regulating p53 expression. This finding is interesting since Bcl6 mediated suppression of p53 (which in turn leads to p21 non expression) has been described as the mechanism of pathogenesis in GCB phenotype of DLBCL.

Paradoxically, this study has shown loss of p21 expression in patients with ABC phenotype to have statistically significant decrease in 5 years survival of 37.2% (p = 0.029**) (Figure 24)**. On the contrary, p21 expression in patients with ABC phenotype had statistically significant increase in 5 years survival of 80.8% **(Table 9)**.

Figure 24: Overall survival of combination of p21 / Immunophenotype

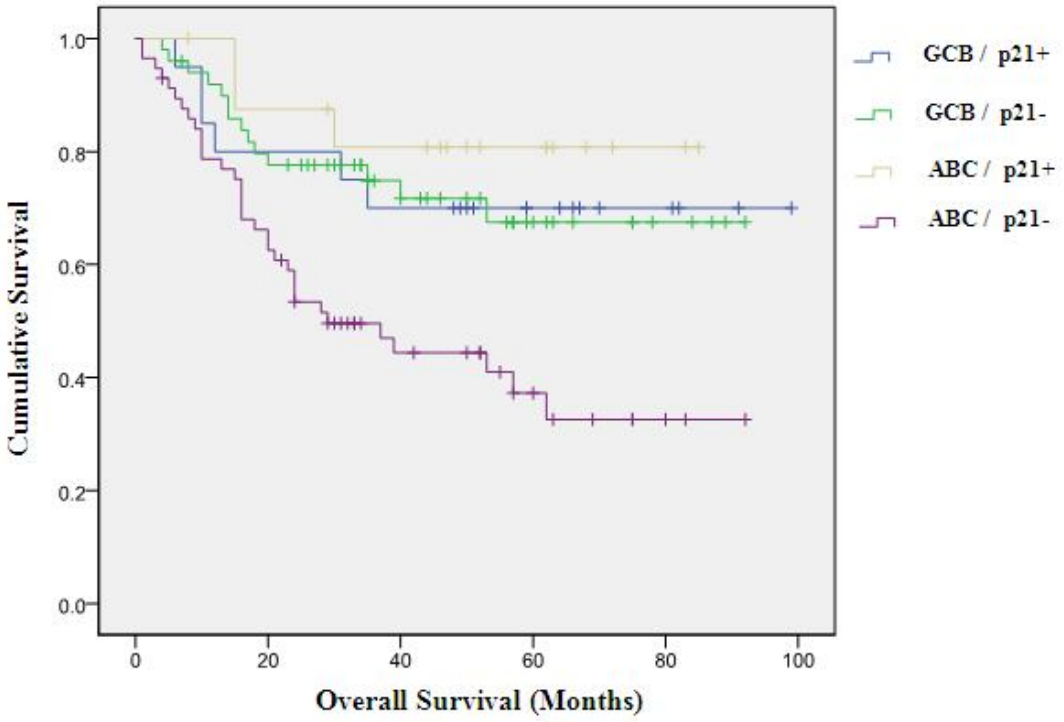


Table 9: Overall Survival of combination of Immunophenotype/p21 expression

Combination	Subgroup (No. of cases)	Overall survival (%)					p-value
		1 yr	2 yr	3 yr	4 yr	5 yr	
GCB / p21+	1 (20)	80.0	80.0	70.0	70.0	70.0	0.001
GCB / p21-	2 (50)	91.9	77.6	74.8	71.7	67.5	
ABC / p21+	3 (17)	100	87.5	80.8	80.8	80.8	
ABC / p21-	4 (57)	78.7	53.4	49.6	44.4	37.2	

Note: 1 Vs 4 (p = 0.023), 2 Vs 4 (p = 0.003), 3 Vs 4 (p = 0.006)

p53 positive cases which included normal and over expression were 107 (74.3%) **(Figure 25)** were compared with p53 negative cases which were 37 (25.7%) for differences in OS. This was not associated with any statistically significant survival outcome **(Figure 26)**. This is because p53 positive cases consists of two subtypes. One

subtype includes normal expression of wild type p53 and the other subtype includes over expression due to mutant p53 identified by the combination of p53 overexpression and p21 negativity (i.e.) $p53^{+++}/p21^{-}$ phenotype. Since there are p53 independent mechanisms of p21 activation, the loss of p53 activity and p21 activity could possibly have additive adverse survival effects.

Figure 25: Immunohistochemical expression pattern of p53

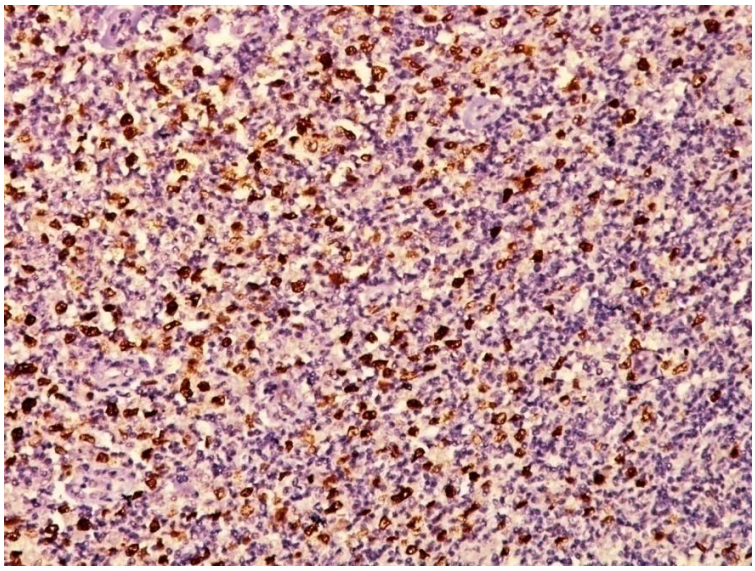
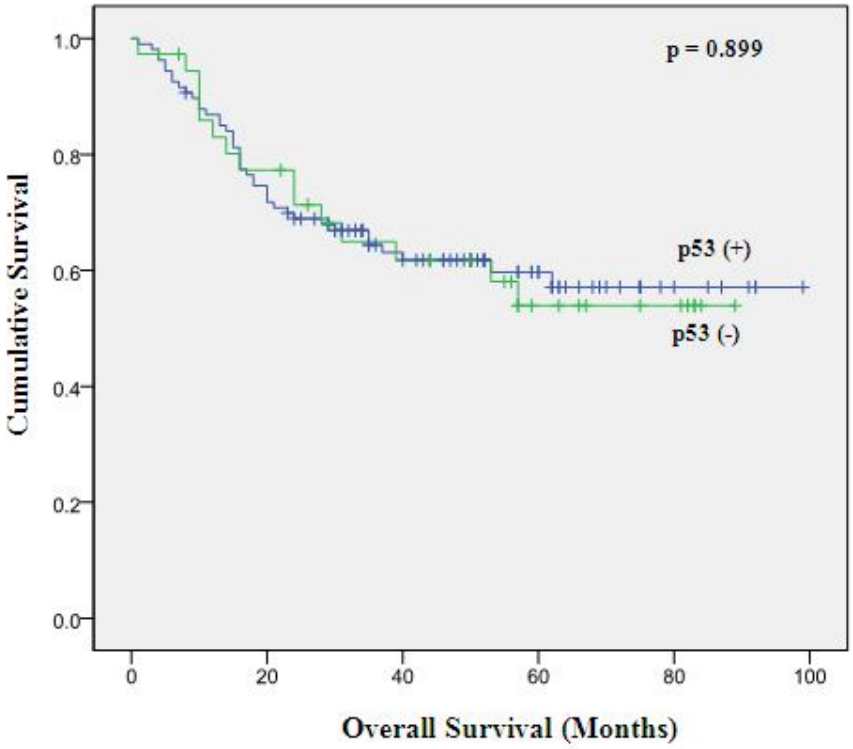


Figure 26 : Kaplan-Meier Overall survival curve for expression of p53

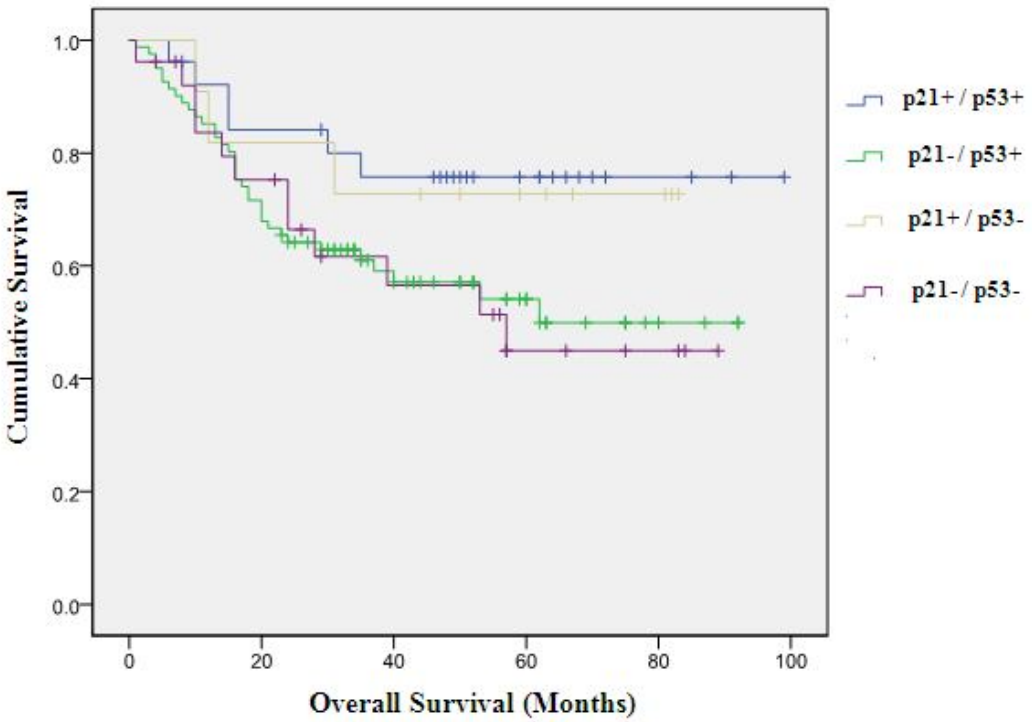


To analyze the complex effect of this heterogenous group, we divided our patients into 4 subgroups based on the combination of p53/p21 expression **(Table 10)** Subgroup 1 (p21+/p53+), subgroup 2 (p21-p53+++), subgroup 3 (p21+/p53-) and subgroup 4 (p21-/p53-). Subgroup 1 (p21+/p53+) had the best 5 years OS of 75.7% but this was not statistically significant **(Figure 27)**. On the contrary, subgroup 4 (p21-/p53-) was associated with poor 5 years OS of 45.0%, but did not reach statistical significance.

Table 10: Overall Survival of combination of p21 / p53 expression

Combination	Subgroup (No. of cases)	Overall survival (%)					p-value
		1 yr	2 yr	3 yr	4 yr	5 yr	
p21 + / p53 +	1 (26)	92.1	84.1	75.7	75.7	75.7	0.169
p21 - / p53 +++	2 (81)	85.2	64.2	61.0	57.1	54.1	
p21 + / p53 -	3 (11)	81.8	81.8	72.7	72.7	72.7	
p21 - / p53 -	4 (26)	83.6	66.4	61.7	56.5	45.0	

Figure 27: Overall survival of combination of p21 / p53 expression



CD10 was positive in 57 cases (39.6%) **(Figure 28)** and negative in 87 cases (60.4%). There appeared to be a trend towards improved 5 years OS in CD10 positive DLBCLs (68.1%) as compared to CD10 negative DLBCLs (57.8%) but was not statistically significant (p = 0.089) **(Figure 29).**

Figure 28: Immunohistochemical expression pattern of CD10

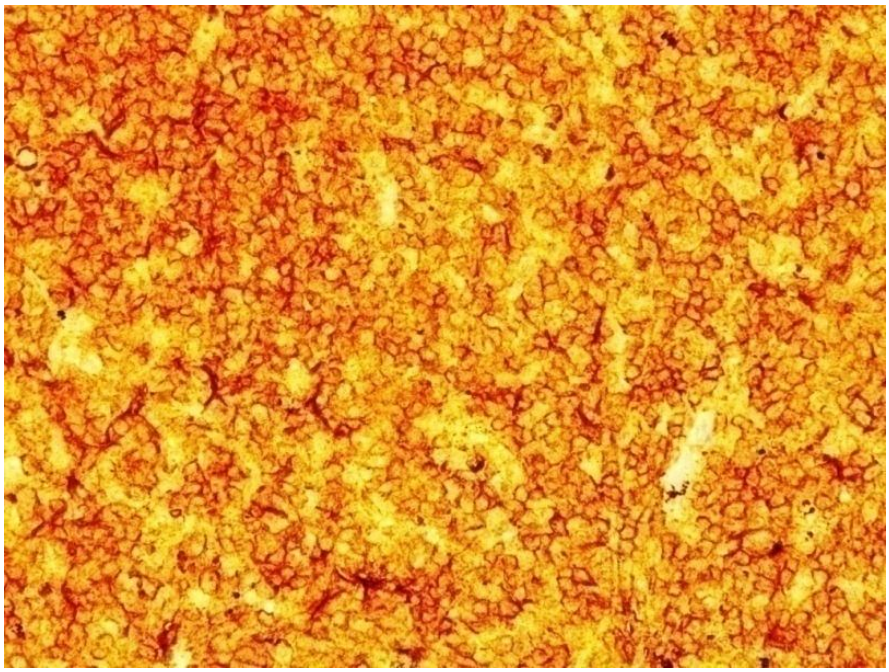
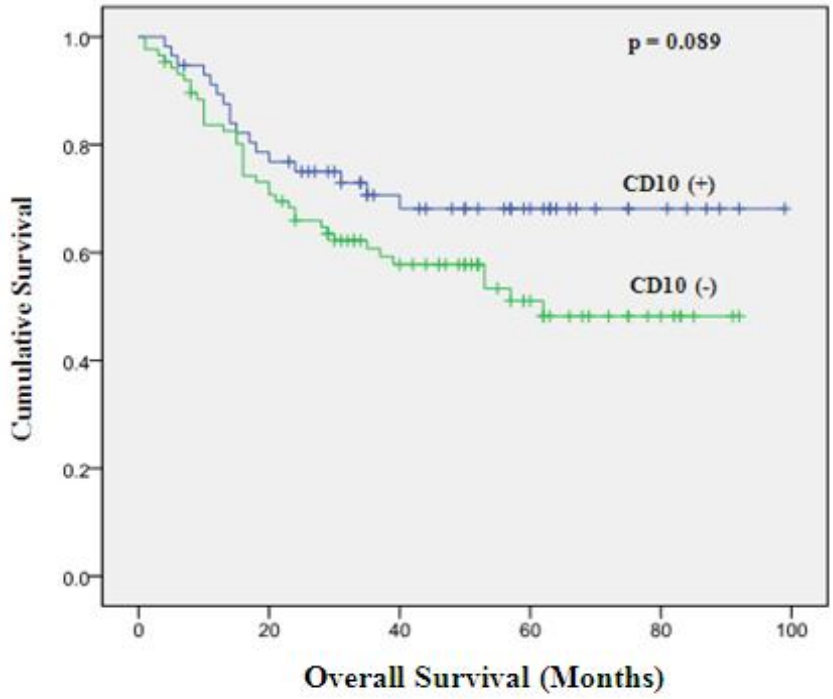


Figure 29 : Kaplan-Meier Overall survival curve for expression of CD10



Bcl6 was positive in 53 cases (36.8%) **(Figure 30)** and negative in 91 cases (63.2%). There was a trend towards improved 5 years survival in Bcl6 positive DLBCLs (66.3%) compared to Bcl6 negative DLBCLs (52.9%) (p = 0.175) **(Figure 31)**.

Figure 30: Immunohistochemical expression pattern of Bcl-6

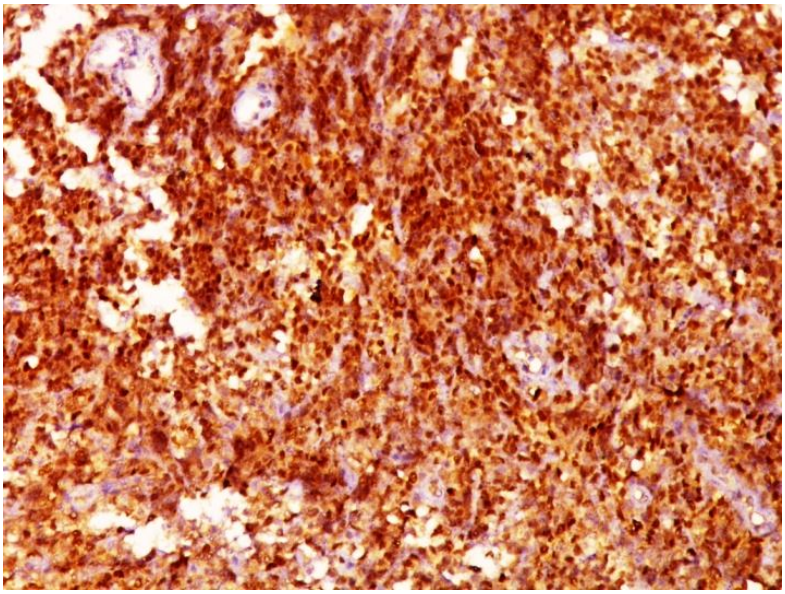
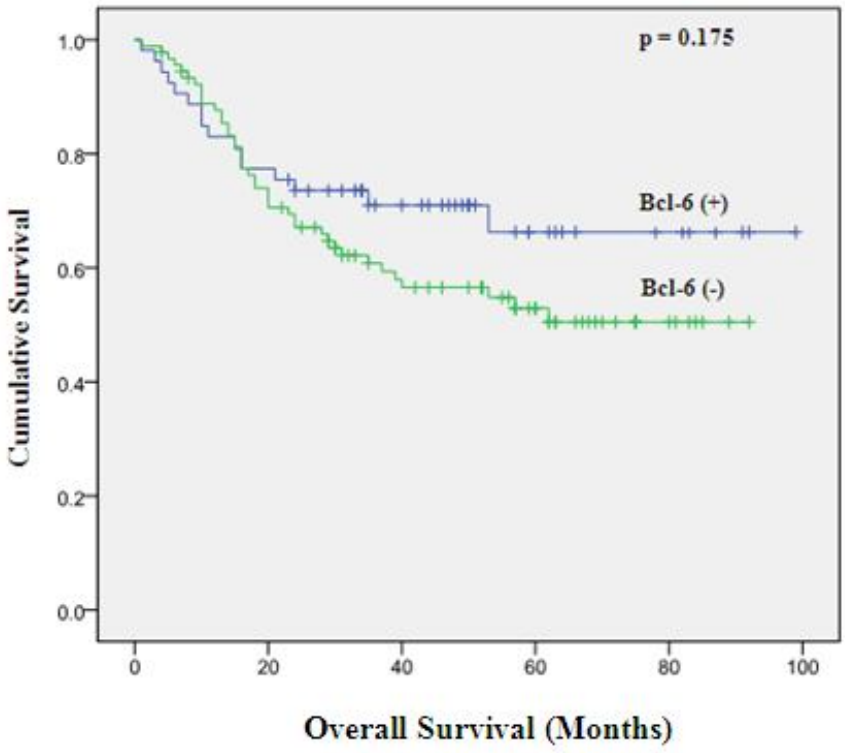


Figure 31 : Kaplan-Meier Overall survival curve for expression of Bcl-6



Mum was positive only in 17 cases (11.8%) **(Figure 32)** and negative in 127 cases (58.2%). The 5 years OS was equal in both groups (58.2%) (p = 0.608) **(Figure 33).**

Figure 32: Immunohistochemical expression pattern of Mum

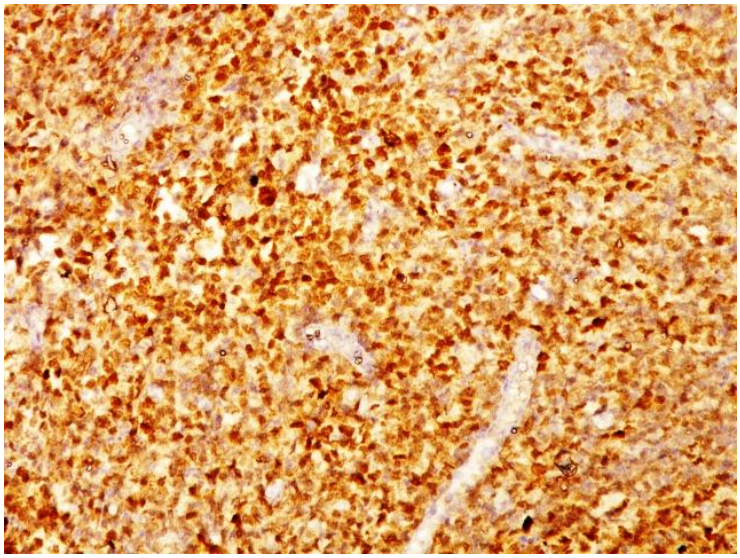
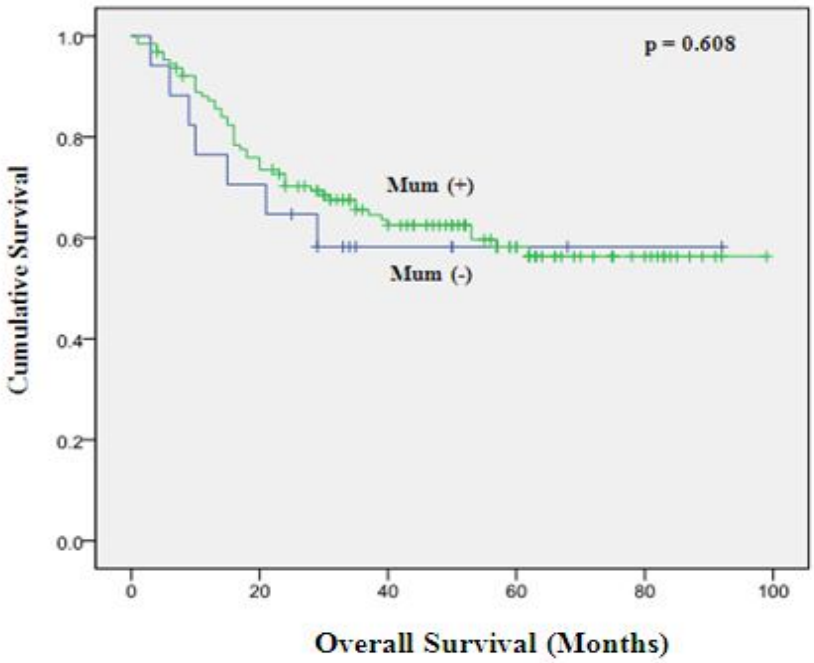


Figure 33 : Kaplan-Meier Overall survival curve for expression of Mum



Blimp was positive only in 5 cases (3.5%) **(Figure 34)** and negative in 139 cases (96.5%). Blimp positivity was associated with a dismal 5 year OS of 20% whereas Blimp negativity was associated with 5 year OS of 60% (p = 0.106). This finding was not statistically significant probably because of very low numbers of Blimp positive cases **(Figure 35)**.

Figure 34: Immunohistochemical expression pattern of Blimp

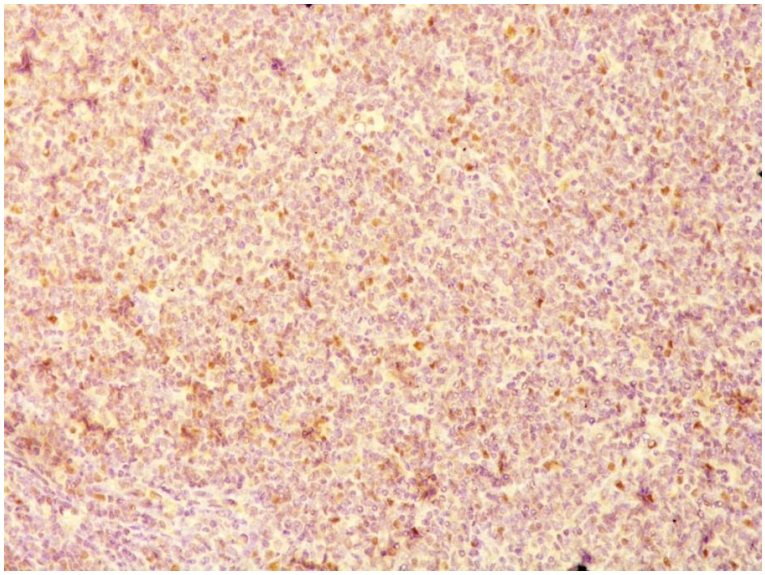
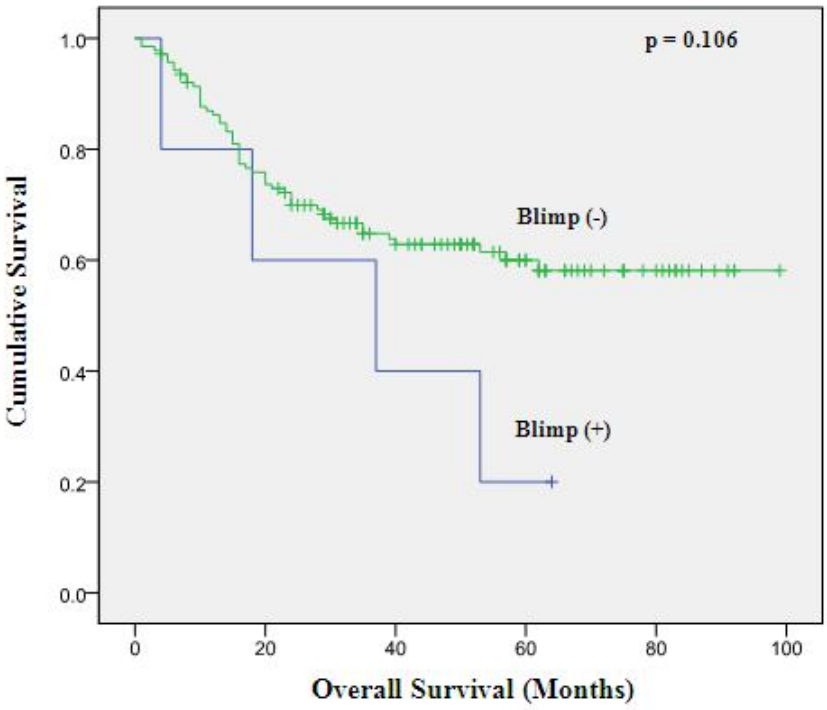


Figure 35 : Kaplan-Meier Overall survival curve for expression of Blimp



Mdm2 was positive in 138 cases (95.8%) **(Figure 36)** and negative in 6 cases (4.2%). Mdm2 positivity was associated with a 5 year OS of 15.9% while Mdm2 negativity was associated with 5 year OS of 33.3% (p = 0.103) **(Figure 37)**.

Figure 36: Immunohistochemical expression pattern of Mdm2

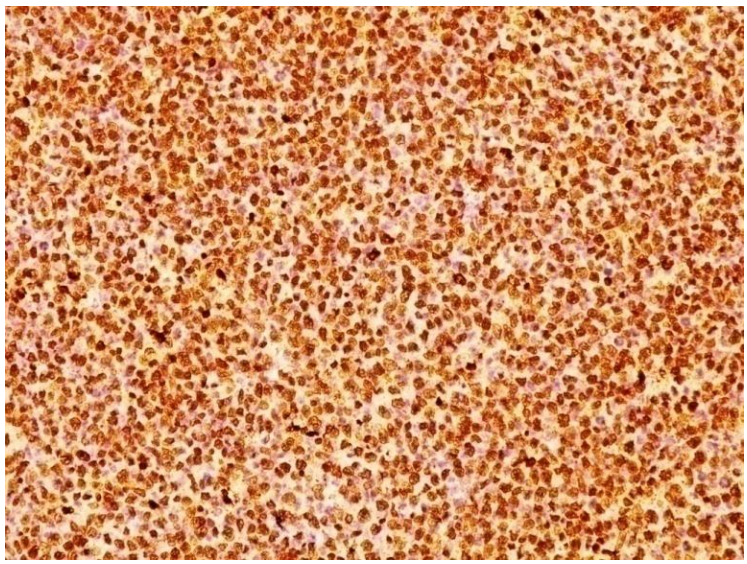
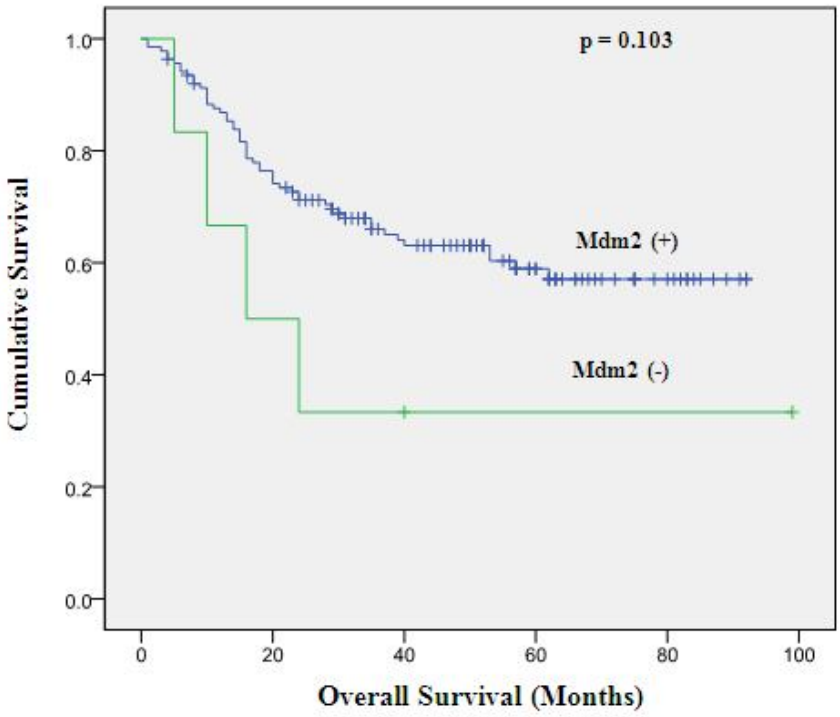


Figure 37: Kaplan-Meier Overall survival curve for expression of Mdm2



p14 was positive only in 71 cases (49.3) **(Figure 38)** and negative in 73 cases (50.7). The overall 5 years survival in p14 positive and negative cases were almost equal at 58.7% and 58.8% respectively (p = 0.325) **(Figure 39)**.

Figure 38: Immunohistochemical expression pattern of p14

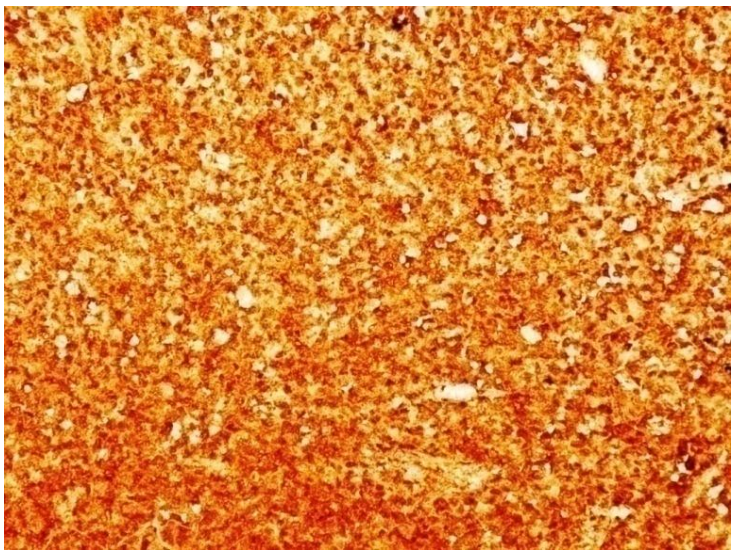
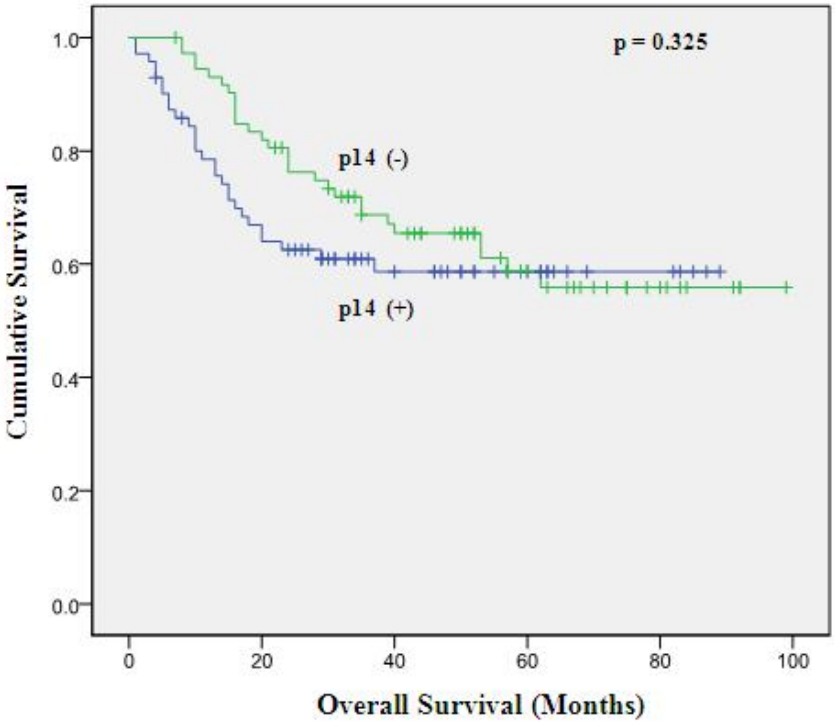


Figure 39 : Kaplan-Meier Overall survival curve for expression of p14

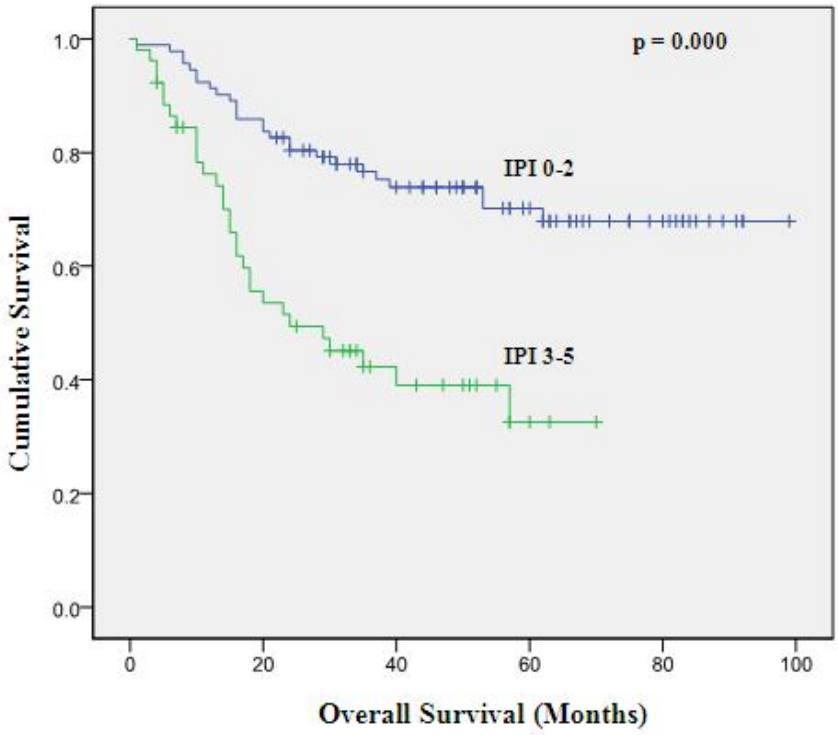


IPI status of 0-2 (92 cases) was associated with a 5 year OS of 70.2% compared to 32.6% survival of IPI 3-5 (52 cases). This finding was statistically very significant (p= 0.000) reiterating IPI status based risk stratification as one of the most powerful tool for pretreatment prognostication **(Figure 40) (Table 11)**.

Table 11: Overall survival based on combination of IPI status

Factor	No. of cases	Overall survival (%)					p-value
		1 yr	2 yr	3 yr	4 yr	5 yr	
IPI status							
0-2	92	91.3	80.4	76.6	73.9	70.2	<0.001
3-5	52	76.2	49.4	42.3	39.1	32.6	

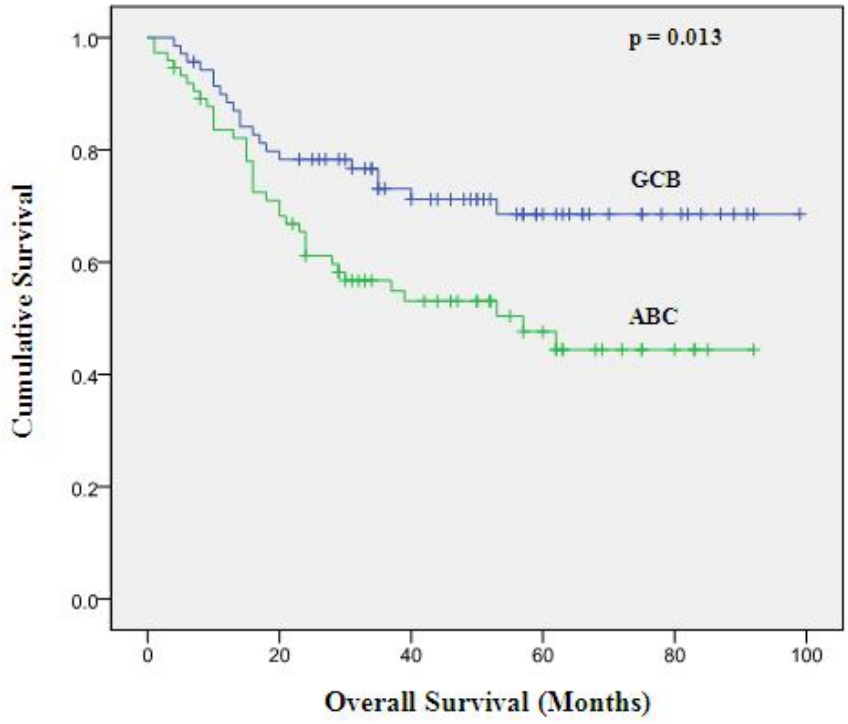
Figure 40 : Kaplan-Meier Overall survival curve for IPI status



Immunophenotype GCB of DLBCL constituted 70 cases (48.6%) whereas ABC constituted 74 cases (51.4%). GCB was associated with a statistically significant higher 5

years OS of 68.6% compared to lower overall 5 years survival of 57.2% in ABC phenotype of DLBCL (p = 0.013) **(Figure 41)**.

Figure 41: Kaplan-Meier Overall survival curve of DLBCL subsets



Ki67 was high in 81 cases (56.3%) **(Figure 42**) and low in 63 cases (43.7%). High Ki67 was associated with a 5 year survival of 59.1%. Low Ki67 was associated with a 5 year survival of 56.8% (p = 0.936). There was no significant difference in OS between the two **(Figure 43)**.

Figure 42: Immunohistochemical expression pattern of Ki-67

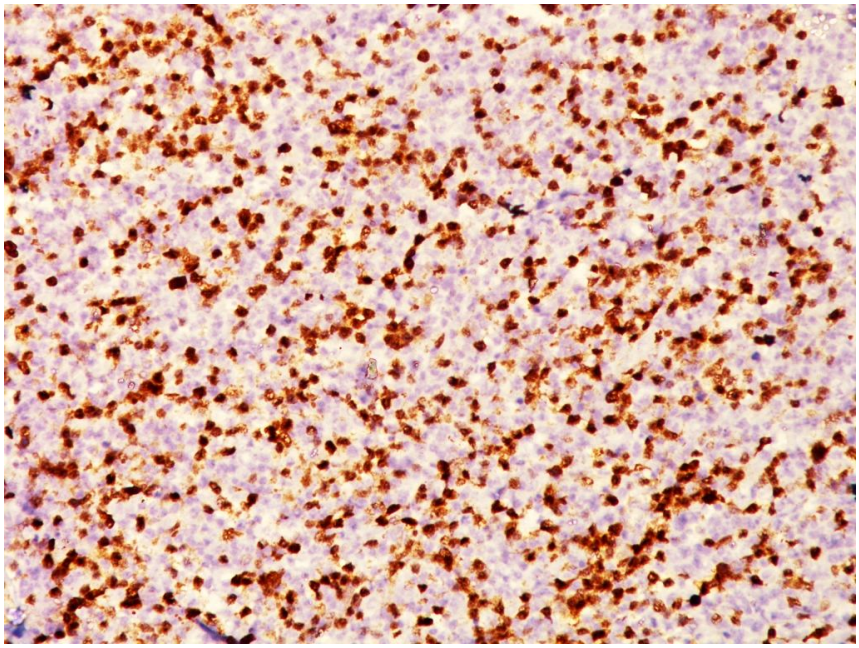
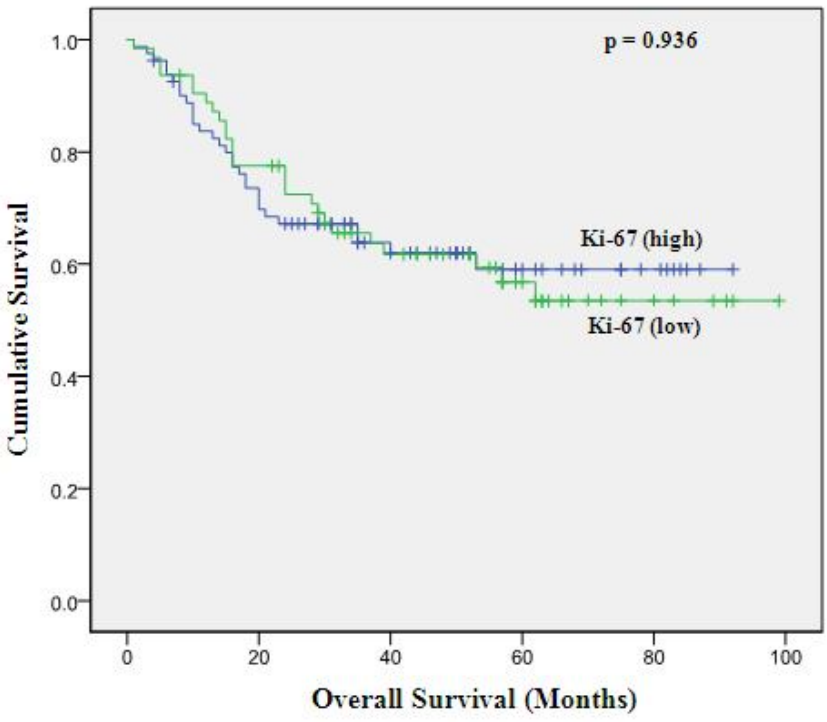


Figure 43: Kaplan-Meier Overall survival curve for expression of Ki-67



All the factors which emerged significant in univariate analysis were subjected to multifactorial analysis by Cox proportional hazard model **(Table 12)**. IPI and Bax/Bcl-2 emerged as independent prognostic factors for OS in DLBCL. 65% decreased risk of death was observed for Bax-/Bcl-2- phenotype compared to Bax+/Bcl-2+ phenotype (p = 0.044). 65% decreased risk of death was observed in low IPI status compared to high IPI status (p = <0.001).

Table 12: Multifactorial analysis of prognostic factors

Factor	No. of cases	Hazard ratio	95% CI	p-value
p21				0.096
Negative	107	1.000	-	
Positive	37	0.541	0.26 – 1.11	
NFkB				0.076
Negative	48	1.000	-	
Positive	96	1.832	0.93 – 3.57	
Immunophenotype				0.297
ABC	74	1.000	-	
GCB	70	1.731	0.40 – 1.32	
BAX/BCL2				0.276
+/+	37	1.000	-	
+/-	29	0.817	0.38 – 1.75	
-/+	50	0.904	0.47 – 1.74	
-/-	28	0.352	0.12 – 0.97#	
IPI				**0.000***
3-5	52	1.000	-	
0-2	92	0.351	0.20 - 0.60$	

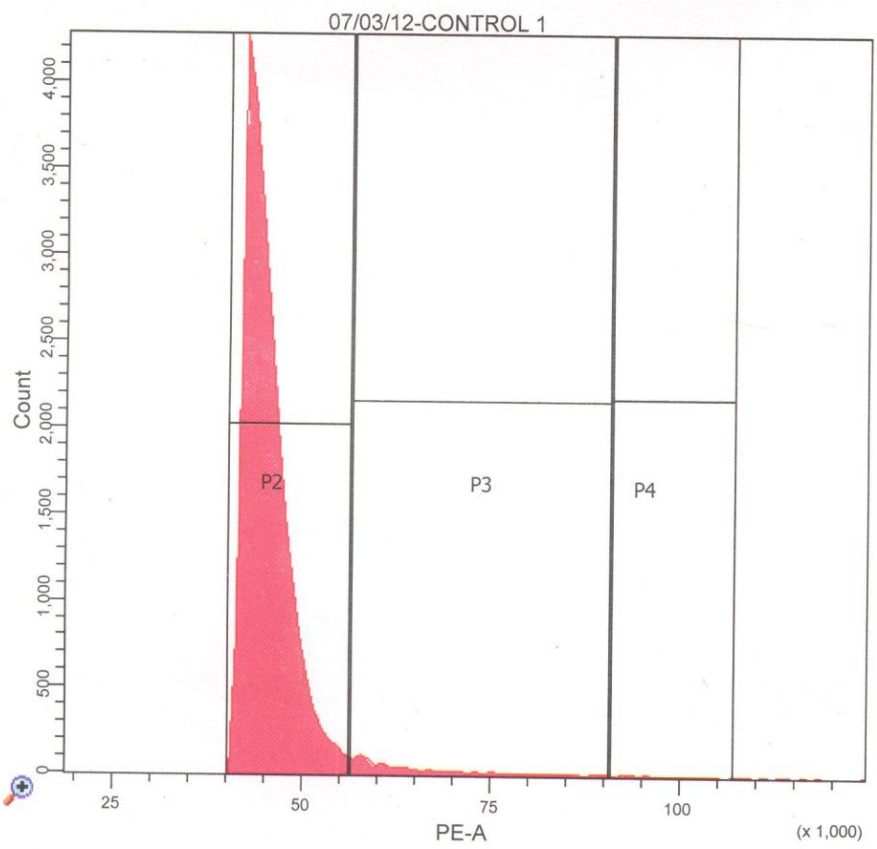
Note: Each factor adjusted for the rest in the table
#p = 0.044, $ p < 0.001

7.2 FLOWCYTOMETRIC ANALYSIS OF CELL CYCLE AND DNA INDEX

Out of the 55 cases studied 33 cases had an increased S-phase fraction and 22 cases had a decreased S-phase fraction with 5 year overall survival of 54.4% and 57.3% respectively **(Table 13)**. However this was not statistically significant (p = 0.977).

DNA Index was diploid in 9 cases , hypoploid in 8 cases and hyperploids in 38 cases with an overall 5 year survival of 37%, 60% and 58%, respectively **(Figure 44 a-d)** . This failed to achieve statistical significance (p = 0.502) **(Table 14)**. These results could be due to the low number of the samples studied.

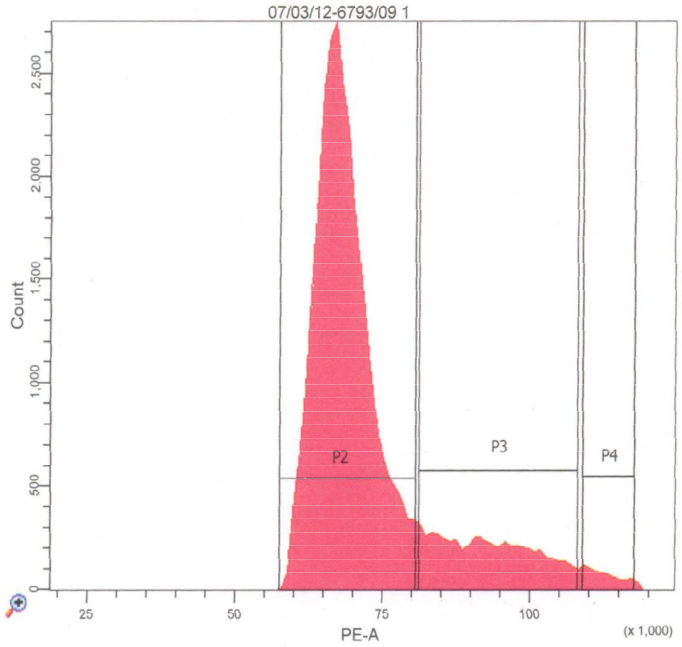
Figure 44a: Control pattern of DNA Index



Experiment Name: LYMPHOMA
Specimen Name: 07/03/12
Tube Name: CONTROL 1

Population	#Events	%Parent	PE-A Mean
P1	23,542	10.8	46,925
P2	22,270	94.6	45,575
P3	1,096	4.7	67,218
P4	109	0.5	96,588

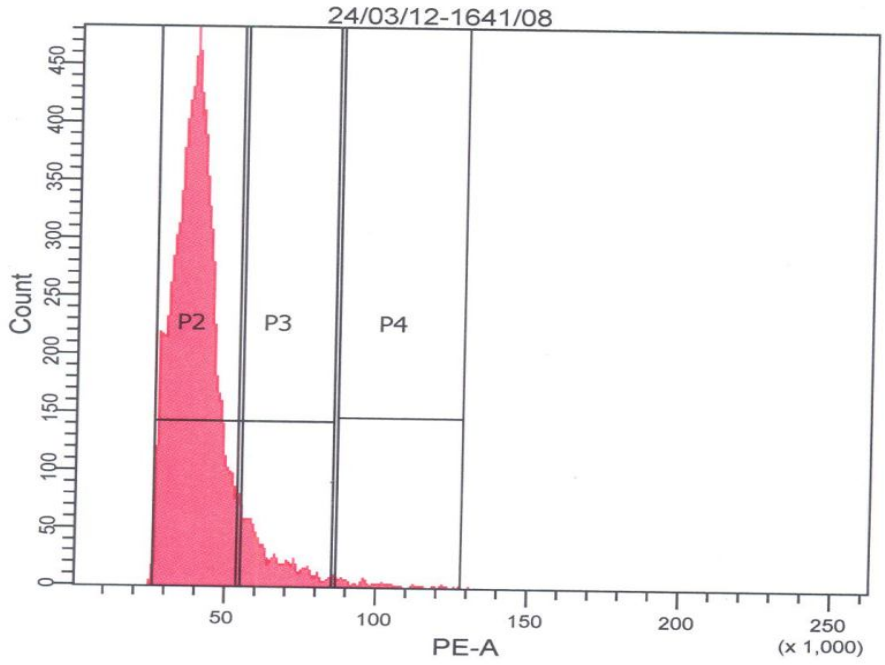
Figure 44b: Hyperploid pattern of DNA Index



Experiment Name: LYMPHOMA
Specimen Name: 07/03/12
Tube Name: 6793/09 1

Population	#Events	%Parent	FSC-A Geo Mean	PE-A Mean
P1	33,497	8.2	256,713	73,720
P2	26,959	80.5	255,960	68,530
P3	5,599	16.7	259,911	93,009
P4	658	2.0	259,578	112,498

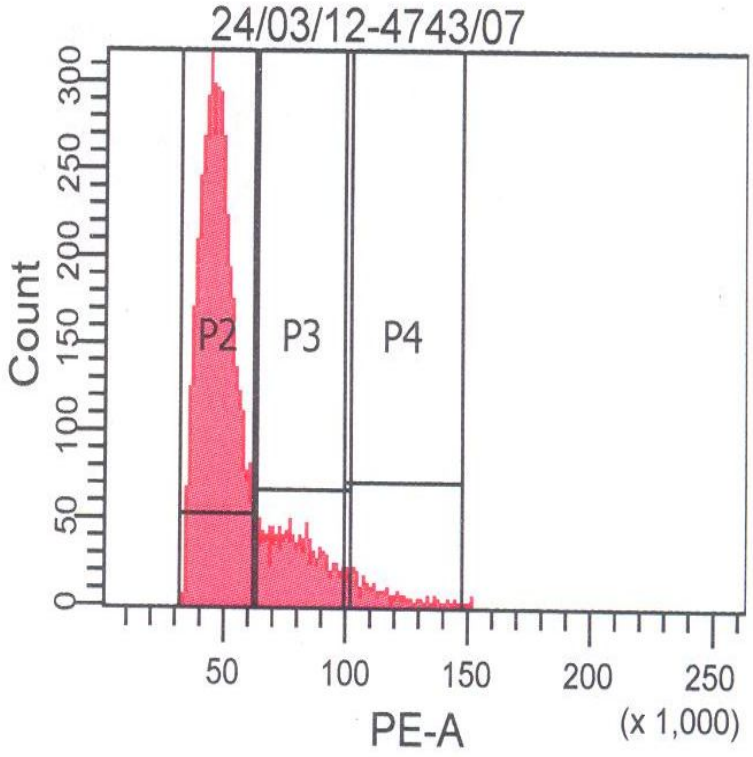
Figure 44c: Hypoploid pattern of DNA Index



Experiment Name: LYMPHOMA
Specimen Name: 24/03/12
Tube Name: 1641/08

Population	#Events	%Parent	PE-A Mean
P1	7,923	8.5	41,638
P2	7,134	90.0	38,635
P3	788	9.9	63,794
P4	78	1.0	99,678

Figure 44d: Diploid pattern of DNA Index



Experiment Name: LYMPHOMA
Specimen Name: 24/03/12
Tube Name: 4743/07

Population	#Events	%Parent	PE-A Mean
P1	6,537	7.4	54,992
P2	5,097	78.0	46,050
P3	1,252	19.2	79,341
P4	287	4.4	113,199

Table 13: Overall survival in patients with increased and decreased S-phase fractions

S-phase fraction	Number of Cases	Overall survival (%)					p-value
		1 yr	2 yr	3 yr	4 yr	5 yr	
>10	33	87.6	75.1	67.3	62.1	54.4	0.977
<10	22	86.4	63.6	63.6	57.3	57.3	

Table 14: Overall survival in patients with Diploid, Hypoploid and Hyperploid DNA Index

DNA Index	Number of Cases	Overall survival (%)					p-value
		1 yr	2 yr	3 yr	4 yr	5 yr	
Diploid	9	77.8	55.6	37.0	37.0	37.0	
Hypoploid	8	87.5	75.0	60.0	60.0	60.0	0.502
Hyperploid	38	89.3	73.0	73.0	64.4	58.0	

7.3 P53 MUTATION

P53 gene mutation analysis was done on 40 cases where fresh samples were available. P53 gene mutation analysis was done to determine the molecular heterogeneity of p53 mutant phenotype group. This was also done to determine whether IHC based mutant phenotype profile expression (p53+++/p21-) was a reliable surrogate marker for mutation of P53 gene associated with its functional loss.

The single nucleotide changes found in the intronic regions of TP53 gene are shown in **Table 15**. Seven polymorphisms were found in exons 2, 4, 7 and 10 respectively **(Figure 45)**. The details of the polymorphisms are shown in **Table 16**.

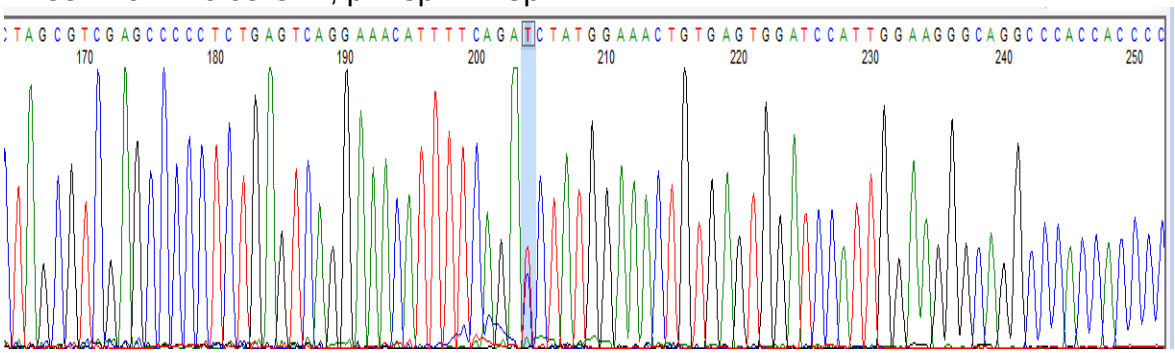
The missense mutations present in exon 7 and 10 were found to show deleterious effect by SIFT database. All the seven polymorphisms were cross checked in IARC TP53 database. The amino acid change M237I and R248W was found to fall in the DNA binding domain region of the TP53 gene and the residue R248W was found to be involved in direct DNA-protein interaction.

All seven polymorphisms present in 27 cases were compared with the immunohistochemical expression of p53 and 21 proteins **(Table 17)**. All 27 patients should have shown the P53 mutant phenotype (p53+++/p21-) but only 22 out of the 27 cases showed the P53 mutant phenotype (p53+++/p21-). The remaining 5 cases which did not have the p53 mutant phenotype (p53+++/p21-) had the Arg 72 Pro change, which was found to be a common polymorphism. Hence this study of p53 gene mutation analysis has proven that IHC based mutant phenotype profile expression (p53+++/p21-) is not a reliable surrogate marker of P53 gene mutation associated functional loss.

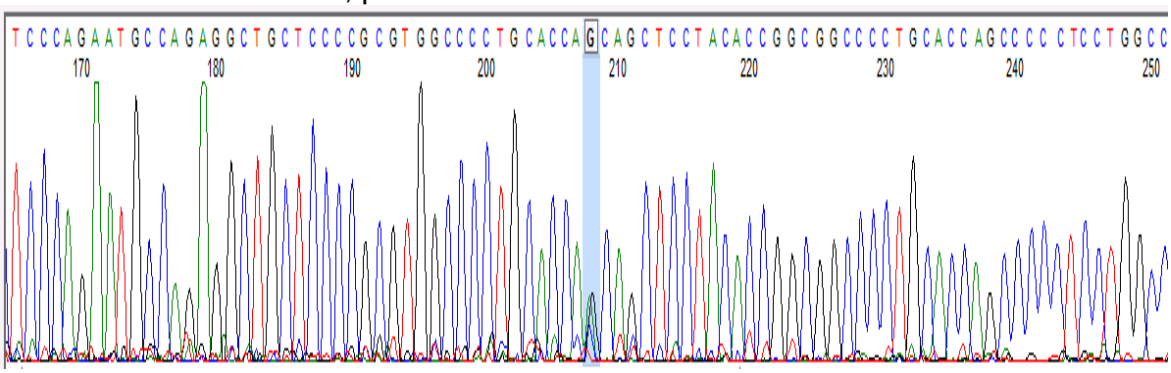
Out of 40 cases studied for p53 gene mutation, 27 cases (67.5%) showed mutations. Among them 15 cases (55.5%) were dead and only 12 (44.5%) were alive. This was not statistically significant due to the small sample size.

Figure 45: TP53 Polymorphisms in Exonic region

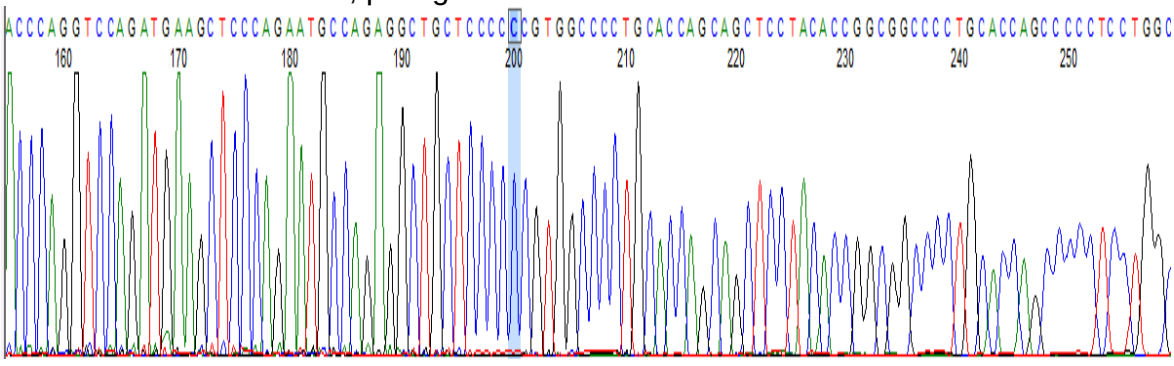
TP53 Exon 2: c.63 C>T; p. Asp 21 Asp



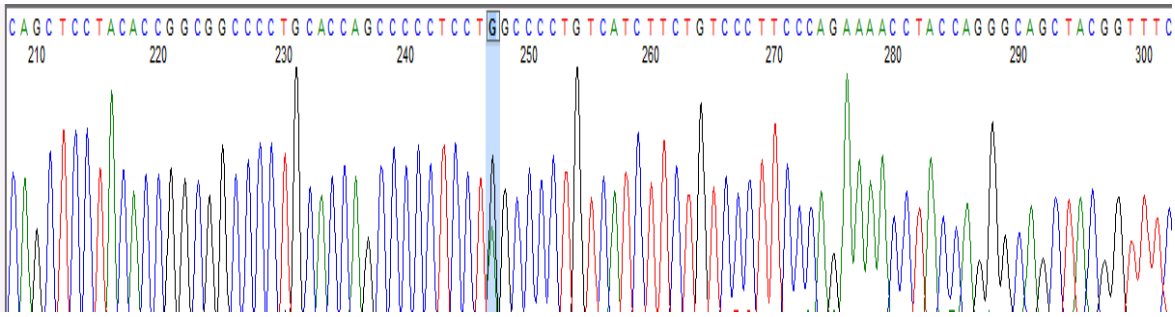
TP53 Exon 4: c.232 G>A; p. Ala 78 Thr



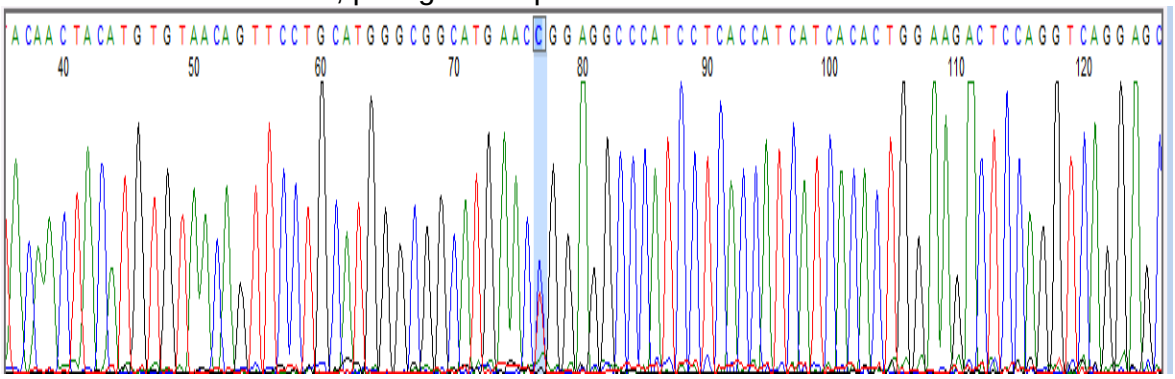
TP53 Exon 4: c.215 G>C; p. Arg 72 Pro



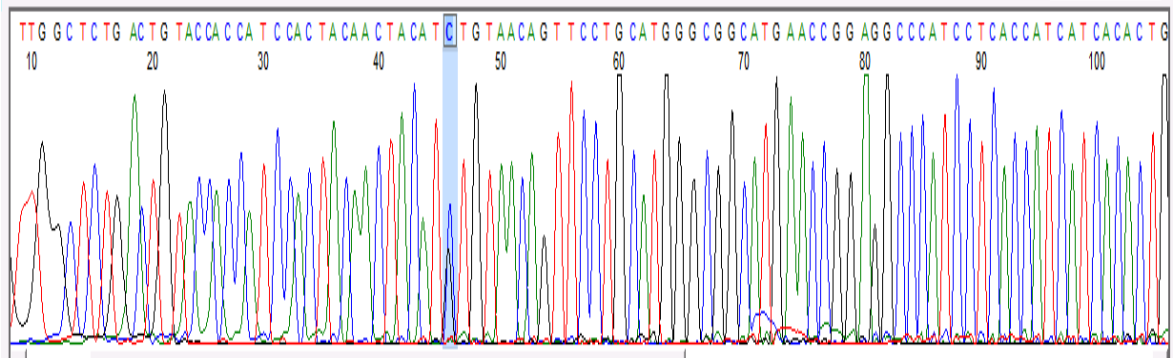
TP53 Exon 4: c.272 G>A; p. Trp 91*



TP53 Exon 7: c.742C>T; p. Arg 248 Trp



TP53 Exon 7: c.711 G>C; p. Met 237 Ile



TP53 Exon 10: c. 1015G>A; p. Glu 339 Lys

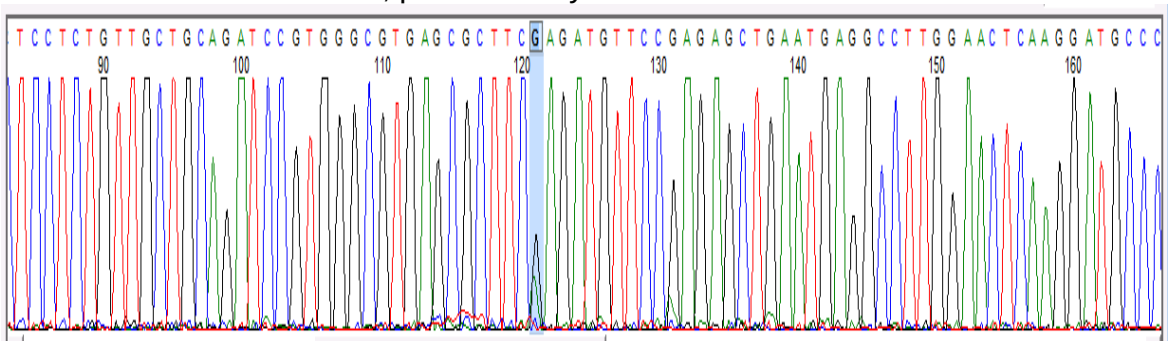


Table 15: TP53 polymorphisms in Intronic region

Exon	Nucleotide	Designation	Cases (n=40) in percentage (%)	Effect
2	c.74+38 G>C	IVS 2 +38 G>C	25 (62.5%)	Unknown
2	c.74+75 G>A	IVS 2 +75 G>A	1 (2.5%)	Unknown
6	c.671 +15 T>A	IVS 6 +15 T>A	2 (5.0%)	Unknown
6	c.671 +17 G>A	IVS 6 +17 G>A	1 (2.5%)	Unknown

Table 16: TP53 Polymorphisms in Exonic region

Exon	cDNA region	Protein region	Effect	Cases (n=40) in percentage (%)	SIFT effect
2	c.63C>T	p.D21D	silent	1 (2.5%)	NA
4	c.232G>A	p.A78T	missense	1 (2.5%)	neutral
4	C.272G>A	p.W91*	nonsense	1 (2.5%)	NA
4	c.215G>C	p.R72P	missense	22 (55.0%)	NA
7	c.742C>T	p.R248W	missense	2 (5.0%)	deleterious
7	c.711G>C	p.M237I	missense	2 (5.0%)	deleterious
10	c.1015G>A	p.E339K	missense	1(2.5%)	deleterious

Table 17: Comparison of sequencing results with IHC results

S.No	Protein change	Mutation effect	IHC result		Immuno-phenotype	Alive/ Dead
			P53 status	P21 status		
1	Arg 72 Pro	Missense	Positive	Negative	GCB	Alive
2	Ala 78 Thr	Missense	Positive	Negative	ABC	Dead
3	Arg 72 Pro	Missense	Positive	Negative	ABC	Alive
4	Arg 72 Pro	Missense	Positive	Negative	GCB	Dead
5	Asp 21 Asp	Silent	Positive	Negative	GCB	Dead
6	Trp 91 *, Arg 248 Trp	Nonsense, Missense	Positive	Negative	ABC	Dead
7	Arg 72 Pro	Missense	Positive	Negative	GCB	Alive
8	Glu 339 Lys	Missense	Positive	Negative	ABC	Alive
9	Arg 72 Pro	Missense	Positive	Negative	GCB	Dead
10	Arg 72 Pro	Missense	Positive	Negative	ABC	Dead
11	Arg 72 Pro	Missense	Negative	Negative	GCB	Dead
12	Arg 72 Pro, Arg 248 Trp	Missense	Positive	Negative	GCB	Alive
13	Arg 72 Pro	Missense	Positive	Negative	ABC	Dead
14	Arg 72 Pro	Missense	Negative	Negative	ABC	Dead
15	Arg 72 Pro	Missense	Negative	Negative	ABC	Dead
16	Arg 72 Pro	Missense	Positive	Negative	ABC	Dead
17	Arg 72 Pro	Missense	Negative	Positive	GCB	Dead

18	Arg 72 Pro	Missense	Negative	Negative	ABC	Alive
19	Arg 72 Pro, Met 237 Ile	Missense	Positive	Positive	GCB	Dead
20	Arg 72 Pro	Missense	Positive	Negative	ABC	Alive
21	Arg 72 Pro	Missense	Positive	Negative	GCB	Dead
22	Arg 72 Pro	Missense	Positive	Negative	ABC	Alive
23	Arg 72 Pro	Missense	Positive	Negative	ABC	Dead
24	Arg 72 Pro	Missense	Positive	Negative	ABC	Alive
25	Met 237 Ile	Missense	Positive	Negative	ABC	Alive
26	Arg 72 Pro	Missense	Positive	Negative	ABC	Alive
27	Arg 72 Pro	Missense	Positive	Negative	GCB	Alive

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CHAPTER 8

SUMMARY AND CONCLUSION

The molecular characterization of DLBCL and the study of various cell cycle regulatory and apoptotic proteins showed statistically significant influence on prognosis only in IPI status based stratification, immunophenotypic classification (GCB/ABC), p21 expression based classification and NFkB expression based classification.

The other cell cycle regulatory proteins were associated with trends consistent with conventional published studies but failed to demonstrate any statistically significant conclusions. An increase in sample size may yield statistically significant results. This could also be due to the confounding effects of various other cell cycle regulatory proteins as DLBCL is a complex multihit disease with alteration of multiple survival pathways resulting in multiple drivers of tumour cells proliferation and survival.

CHAPTER 9

RECOMMENDATIONS

1. Immunohistochemistry based molecular profiling of DLBCL along with immunophenotypic classification of the GCB and ABC subtypes of DLBCL must be done routinely in all cases as part of pretreatment workup in order to identify the poor prognostic groups as candidates for early aggressive multimodal therapy.

2. P53 gene mutational studies is preferable over IHC based p53 status as this study has shown IHC based profiling of mutant phenotype (p21-/p53+++) as an unreliable surrogate marker for P53 missense mutations resulting in it's functional loss.

Further molecular studies in NFkB pathways and p21 related pathways might provide more information and possibly identify targets for therapy.

3. This study identified p21 and NFkB as the only two statistically significant variables which can be used to predict prognosis in DLBCL. Expression of NFkB in ABC phenotype identified patient group with the worst 5 years OS of 39.6%. Similarly loss of p21 expression in ABC phenotype identified the patient group with worst 5 years OS of 37.2%. Based on the differential strength of association of various molecular profile groups with prognosis, a differential point based prognostic score system should be developed to better stratify high risk groups.

4. Further molecular studies in NFkB pathways and p21 related pathways might provide more information and possibly identify targets for therapy.

5. Bcl6 is being extensively studied for molecularly targeted therapy. Hence further studies on molecular characterization of Bcl6 and its sub classification to identify a subgroup that is exquisitely bcl6 dependent DLBCL could help in identifying candidates for clinical trials involving Bcl6 inhibitors.

6. To conduct a study with larger sample size to determine if the trends seen in the present study would be amplified to reach clinical significance.

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