# Alterations of Tumour Protein 53 (TP53) Pathways in Diffuse Large B-cell Lymphomas

Thesis submitted for the degree of Doctor of Philosophy

at the University of Leicester

by

Dalia Magdi Abdel azim

M.B,B.Ch; MSc (Clinical Pathology)

Department of Cancer Studies and Molecular Medicine

University of Leicester

April 2009





ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

*To My Family*

# Abstract

## Alterations of Tumour Protein 53 (TP53) Pathways in Diffuse Large B-cell Lymphomas

Dalia Magdi Abdel Azim

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoma. It has heterogeneous clinical features and varies markedly in response to treatment and in prognosis. Although the clinical variables widely used in clinical practice may be useful indicators in defining the underlying biological heterogeneity of DLCL, a search for molecular expression, such as TP53, would improve the prognostic predict ability and current understanding of the clinical behaviour of DLBCL.

Seventy-two cases of DLBCL were assessed by immunohistochemistry using primary antibodies for TP53, p21, HDM2, Phospho-TP53, Bcl-2, Bcl-6 and MIB-1. There was a significant positive correlation between high proliferation index and TP53, phopho-TP53 and Bcl-6 expressions ($p<0.005$), while a significant negative correlation was found with Bcl-2 expression ($p<0.005$). Bcl-2 expression showed an inverse correlation with TP53, phospho-TP53, Bcl-6 ($p<0.005$). TP53 assessed using the DO-1 antibody showed a significant correlation with phopho-TP53 ($p<0.005$). p21 showed a similar correlation with HDM2 ($p<0.005$).

TP53 mutations were assessed using PCR-SSCP analysis in 64 cases of DLBCL. The frequency of TP53 mutations in this study was 18.75%. Exon 7 harboured most mutations (30%). There was a significant correlation between low expression of TP53 and phospho-p53 assessed by immunohistochemistry and the presence of WT-p53, but not vice versa. Also, a significant correlation was found between missense TP53 mutations and TP53+/p21- phenotype ($p<0.005$). High proliferation index correlated with TP53 mutations ($p<0.005$). There was a strong association of the presence of TP53 mutations and low level of miR-34a ($p<0.05$). A strong correlation between miR-34a expression levels and TP53, p21 and HDM2 ($p <0.05$).

In conclusion, although 80% of DLBCL cases posses WT-p53 gene , not of them are fully functional and they show phenotypic variabilty in their TP53 activity. Other mechanisms for inactivating TP53 must be responsible, such as alterations of the downstream or upstream regulators or targets that will mimic a TP53 aberration.

*What we call the beginning is often the end*
*And to make an end is to make a beginning.*

*We shall not cease from exploration*
*And the end of all our exploring*
*Will be to arrive where we started*
*And know the place for the first time.*

*TS Elliot (1888-1965)*

# Acknowledgements

I would like to thank Dr. Howard Pringle , my main supervisor, for his continuos help, support and endless enthusiam, without your help and ideas this would not be finished. Also my thanks to Dr. Kevin West, consultant Histopathologist at UHL for guiding me through the journey into lymphomas.

I would like to thank also Dr Jacqui Shaw and Prof. Rosemary Walker for the scientific and moral support they have given me. I really appreciate your concern and dedication.

Thanks are due to Lindsay Primrose for the help with PCR , Linda Potter for the help with sequencing and RT-PCR and Angie Gillies for help with immunohistochemistry.

Special thanks to Karen Kulbicki, not only for the techniqual help, but also for the the warmth and care she has always given me.

Lastly for the postdocs and the students, past and present, wishing you all the best and thanks for being there. With the perseverance and hard work you can achieve all your dreams.

Lastly, I would like to thank my mother Prof. Amina Abdel Aal for believing in my intellect and capabilities and also Prof. Nick London and his wife Sue for being a second family away from my home.

## Contents:

## List of Tables

## List of Figures

## List of abbreviations

| | |
|---|---|
| ABC | Avidin Biotin Complex |
| ABC-DLBCL | Activated B-cell-Diffuse Large B-cells lymphoma |
| AP | Alkaline phosphatise |
| Apaf-1 | Apoptotic peptidase activating factor 1 |
| APS | Ammonium Persulphate |
| ATM | Ataxia- Telangectasia mutated |
| ATR | ATM-related peptide |
| Bax | Bcl-2 associated X protein |
| Bak | Bcl-2 homologous killer |
| Bcl-2 | B-cell Leukaemia Lymphoma 2 |
| Bcl-6 | B-cell Leukaemia Lymphoma 2 |
| Bim | Bcl-2 interacting mediator of cell death |
| BL | Burkitts Lymphoma |
| bp | base pair |
| BSA | Bovine Serum Albumin |
| Caspases | cysteine-aspartic acid proteases |
| cDNA | complementary DNA |
| cdk | cyclin-dependent kinase |
| ChIP | Chromatin immunoprecipitation |
| CKI | Cyclin- dependent Kinase Inhibitor |
| CLL | Chronic Lymphocytic Leukaemia |
| DBD | DNA Bindind Domain |
| DNA | Deoxy Nucleic Acid |
| DNA-PK | DNA-dependent Protein Kinase |
| DLBCL | Diffuse Large B-Cell Lymphoma |

| | |
|---|---|
| COP-1 | COnstitutive Photochromogenic 1 |
| dNTP | Doexy nucleotide 5'triphosphate |
| EDTA | Ethylene DiamineTetraAcetic acid |
| FFPE | Formalin-fixed paraffin embedded |
| FL | Follicular Lymphoma |
| GADD45 | Growth Arrest and DNA Damage inducible gene |
| H&E | Haematoxylin and Eosin |
| HDAC | Histone deacetylase |
| HDM2 | HumanDouble Minute clone 2 |
| IAPs | Inhibitor of Apoptosis Proteins |
| IHC | ImmunoHistoChemistry |
| JNK | c-Jun N Terminal Kinase |
| KDa | Kilo Dalton |
| LFS | Li-Fraumeni Syndrome |
| M | Molar |
| MDM2 | Murine Double Minute clone 2 |
| mg | milligram |
| µg | microgram |
| µl | microlitre |
| min | minute |
| mRNA | messenger Ribonucleic Acid |
| miRNA | micro RNA |
| miR-34a | micro-RNA 34a |
| MZL | Mantle Zone Lymphoma |
| NHL | Non-Hodgkin Lymphoma |
| NLS | Nuclear Localisation Signal |
| nt | nucleotides |

| | |
|---|---|
| o/n | Over-night |
| p14/ARF | Protein of 14 kDa/Alternative Reading Frame |
| p21/WAF1 | Protein of 21 kDa/Wild type p53 activated factor 1 |
| p63 | Protein of 63 kDa |
| p73 | Protein of 63 kDa |
| PBS | Phosphate Buffered Saline |
| PCNA | Proliferating cell nuclear antigen |
| PCR | Polymerase Chain Reaction |
| PIG | p53-Inducible gene |
| PIRH-2 | p53-induced RING-H2 domain protein |
| PK | Proteinase K |
| PM-DLBCL | Primary mediastinal-Diffuse large cell lymphoma |
| PR | Proline rich domain |
| Pre.miRNA | Precursor micro RNA |
| Pri-miRNA | Primary micro RNA |
| PUMA | p53 Upregulated Modulator of Apoptois |
| RISC | RNA-induced silencing complex |
| RT-PCR | Reverse Transriptase- Polymerase Chain Reaction |
| RNA | Ribonucleic Acid |
| Sec(s) | second(s) |
| SSCP | Single Strande Conformational Polymorphism |
| TAD | Transactivation Domain |
| TAE | Tris Acetate EDTA |
| Taq | Thermus Aquaticus DNA polymerase |
| TCR | T-Cell rich lymphomas |
| TBE | Tris Borate EDTA |
| TEMED | N,N,N',N'-tetramethylethylenediamine |

| | |
|---|---|
| TE | Tris EDTA |
| TP53 | Tumour suppresso gene |
| TP53 | Tumour Protein of 53 kDa |
| TTD | Tetramerisation domain |
| V | volt |
| WHO | World Health Organistaion |
| WT-p53 | Wild-type p53 |

*To My Family*

# Chapter 1

# Introduction

# 1 Introduction

## *1.1 Lymphomas*

Lymphomas are a heterogeneous group of lymphoid malignancies arising in the lymphoid tissue. They are mostly B-cell in origin with a minority of T-cell origin. Lymphoid neoplasms are the sixth most common group of malignancies worldwide in both men and women (Ferlay, Autier et al. 2007). Some lymphoid neoplasms have been linked to certain infections and immunosuppressive drugs. However, the aetiologies of most lymphoid neoplasms remain largely unknown. The heterogeneous nature of lymphomas and evidence from epidemiologic studies increasingly point to aetiologic heterogeneity among the lymphoid neoplasm subtypes (Rothman, Skibola et al. 2006). Lymphomas are broadly classified into Hodgkin's (HL) and Non- Hodgkin's Lymphomas (NHL).

### 1.1.1 Classification of Lymphomas

The understanding of the immunologic and molecular basis of lymphoid neoplasms have resulted in the evolution of numerous clinical and pathologic classification schemes over the past 50 years, particularly for (NHL). Lymphomas were categorized predominantly by morphology according to the Rappaport classification (Rappaport 1966), by morphology and clinical prognosis according to the Working Formulation (Rosenberg SA 1982) or by cell lineage and differentiation according to Kiel classification (Stansfeld, Diebold et al. 1988). These lymphoma classification schemes were largely replaced in 1994 by the Revised European-American Lymphoma (REAL) classification, which incorporated morphologic, immunophenotypic, genotypic, and clinical features into disease subtype definitions (Herrinton 1998).

In 2001, the World Health Organization (WHO) introduced a new classification built on the REAL classification that represents the current "gold standard" for classifying all haematopoietic neoplasms (Jaffe et al 2001). Within the lymphoid neoplasms, the WHO

system distinguishes Hodgkin lymphoma from NHL based on morphologic and immunologic characteristics. Stage of differentiation and additional morphologic, phenotypic, genotypic, and clinical features are used to distinguish the various NHL subtypes (Table 1-1).

**Table1-1: WHO Classification of Non-Hodgkin Lymphoma.** (From Jaffe et al 2001)

| B-cell neoplasms | T-cell neoplasms |
|---|---|
| ***Precursor B-cell neoplams*** | ***Precursor T-cell neoplasms*** |
| Precursor B-lymphoblastic leukaemia /lymphoma | Precursor T-lymphoblastic leukaemia /lymphoma |
| ***Mature B-cell neoplams*** | ***Mature T-cell neoplams*** |
| B-Chronic Lymphocytic Leukaemia | T-cell prolymphocytic leukaemia |
| B-cell prolymphocytic leukaemia | T-cell large granular lymphocytic lymphoma |
| Lmphoplasmacytic leukaemia /Waldenstrom macroglobulinaemia | Aggressive NK-cell lymphoma |
| Mantle Zone Lymphoma | Adult T-cell leukaemia /lymphoma |
| Hairy cell leukaemia | Extranodal NK/T-cell lymphoma |
| Plasmacytoma | Mycosis Fungoides/Sezary syndrome |
| Marginal Zone Lymphoma | Systemic anablastic large T-cell lymphoma |
| Follicular Lymphoma | Hepatosplenic T-cell lymphoma |
| Mantle zone lymphoma | Angioimmunoblastic T-cell lymphoma |
| Diffuse Large B-cell Lymphoma | Primary cutaneous T-cell lymphoproliferative disorders |
| Burkitt's lymphoma | Enteropathy-type T-cell Lymphoma |
| **Hodgkin Lymphoma** | |
| **Non classic HL** | **Classic HL** |
| Nodular Lymphocyte Predominant HL | Nodular sclerosis |
| | Mixed cellularity HL |
| | Lymphocyte-rich HL |
| | Lymphocyte depleted HL |

### 1.1.2 Epidemiology of Non-Hodgkin Lymphoma (NHL)

The most striking finding in NHL is the unexplained increase in incidence over the past twenty years in the Western world (Alexander, Mink et al. 2007). Age-adjusted incidence rates show steady increase in both genders (Figure1-1) and the rates are consistently higher in males more than females and in whites compared to non-whites. Elsewhere extra-nodal B-cell lymphomas, nodal T-cell lymphomas, 'high grade' lymphoma and follicular lymphoma, are reported to have increased in incidence more than others. In countries where HIV is more prevalent, increases in immunoblastic and Burkitt-like NHL have been striking (Mead and Mason 1983).

Although the mortality of NHL has decreased in children and young adults, the overall mortality has increased over time. The survival rate, however, differ considerably by the histologic subtype. Low grade lymphomas as Follicular lymphoma (FL) and small lymphocytic lymphoma (SLL) have higher rates than intermediate- grade lymphomas such as DLBCL. High grade lymphomas such as CNS lymphoma and Burkitt's lymphoma have the poorest survival rates. Several risk factors are associated with the development of NHL. These include immunodeficincy states, HIV, EBV (Brauninger, Spieker et al. 2003), Helicobacter pylori (Greiner, Marx et al. 1994) and HTLV-1 (Burkitt 1983). Occupational and environmental exposure to pesticides, organic solvents and UV radiation have been also linked to increased NHL risk (Dryver, Brandt et al. 2004).

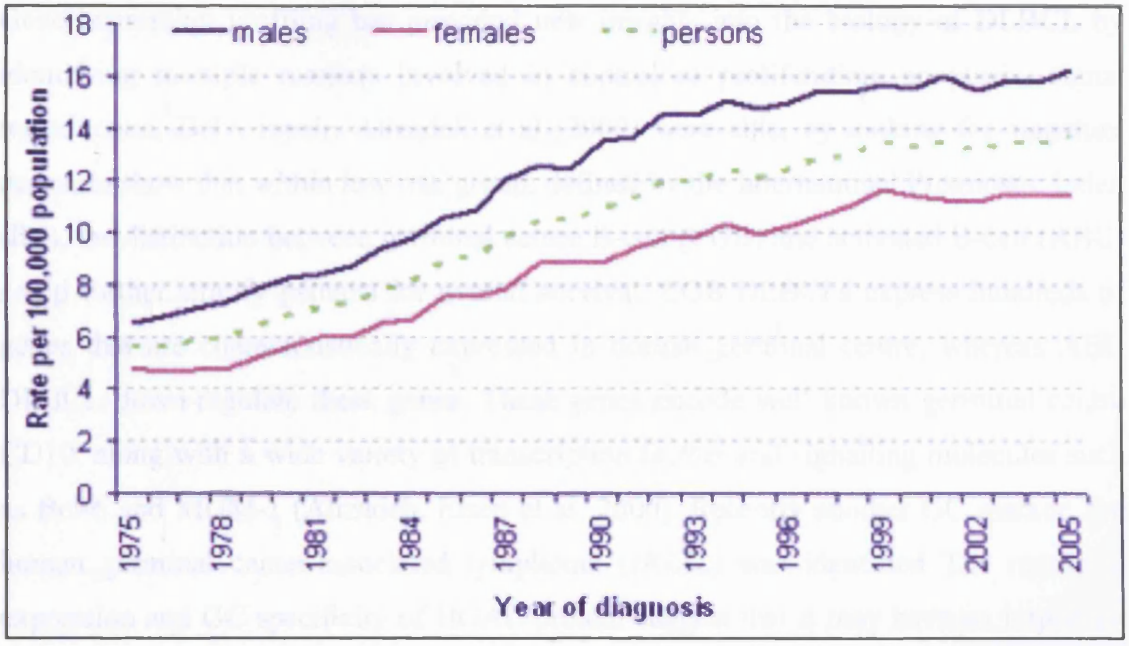



**Figure 1-1: Age-standardised incidence rate for NHL.** From Office for National Statistics. Cancer Statistics registrations: Registrations of cancer diagnosed in 2005, England. Series MB1 no.36. 2008

## *1.2 Diffuse Large B- Cell Lymphoma:*

### 1.2.1 Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most frequent lymphoma in adults worldwide, accounting for 30% to 40% of lymphoid neoplasms (Harris 1997). The diversity in clinical presentation and outcome, as well as the pathologic and biologic heterogeneity, suggest that DLBCL comprises several disease entities that may require different therapeutic approaches. Although DLBCL is a potentially curable disease, only 40 to 50 % of patients can be cured by anthracycline-based combination chemotherapy, whereas the remainder succumbs to this disease, indicating that the therapy should be tailored according to the genetic profile of individual patients (Wright, Tan et al. 2003).

Gene expression profiling has provided new insights into the biology of DLBCL by identifying multiple markers involved in control of proliferation, apoptosis, signal transduction, DNA repair. Alizadeh et al (2000) were able, by looking for signature genes, to show that within low risk group, defined by the International Prognostic Index (IPI), the distinction between germinal centre B-cell (CGB) and activated B-cell (ABC) could further stratify patients for overall survival. CGB DLBCLs express hundreds of genes that are characteristically expressed in normal germinal centre, whereas ABC DLBCL down-regulate these genes. These genes encode well known germinal centre CD10, along with a wide variety of transcription factors and signalling molecules such as Bcl-6 and MUM-1 (Alizadeh, Eisen et al. 2000). Recently another GC marker, the human germinal center-associated lymphoma (*HGAL*) was identified The restricted expression and GC specificity of HGAL protein suggest that it may have an important role in the diagnosis and subclassification of lymphomas and in the identification of subtypes associated with different prognoses as well (Natkunam, Lossos et al. 2005).

Gene-expression profiling has identified three major subgroups of DLBCL, termed germinal centre B-cell-like DLBCL (GCB-DLBCL), activated B-cell-like DLBCL (ABC-DLBCL), and primary mediastinal DLBCL (PM-BCL). These three subgroups of DLBCL are associated with a widely disparate clinical outcome with 5-year survival rates of 59%, 30%, and 64% in patients with GCB-DLBCL, ABC-DLBCL, and PMBCL respectively (Alizadeh AA et al. 2000, Rosenwald A 2003). In addition, GCB-DLBCL is characterized by frequent REL amplifications, BCL2 translocations, and ongoing somatic hypermutation of the immunoglobulin genes (Lossos, Alizadeh et al. 2000). In contrast, ABC-DLBCL and PMBCL have a constitutive activation of the nuclear factor B (NF-B) pathway that they require for survival, which is not a feature of GCB-DLBCL (Davis, Brown et al. 2001).

### 1.2.2 Clinical Presentation

DLBCL, as with other types of NHL, is most commonly presented with painless lymphadenopathy. Approximately 25% of patients present with anatomically limited disease, while the remaining 75% having more advanced disease. Many patients experience constitutional (B) symptoms. These include night sweats, night fevers and unexplained weight loss. Extra-nodal involvement is quite common and almost any organ can be affected by the disease (Oudejans, van Wieringen et al. 2009).

Several clinical parameters have been shown to be significantly associated with outcome. These estimate the disease burden and the patient characteristics. The IPI model was established in the early 1990s and it is still the best validated and most successful prognostic tool. IPI comprises five elements: age, stage of disease, performance status, extra-nodal sites and LDH levels. Despite the prognostic value of IPI, it is purely based on the ability to predict clinical outcome without providing insight into the biologic heterogeneity of DLBCL (Wilder, Rodriguez et al. 2002).

### 1.2.3 Pathology

In DLBCL, as the name implies, malignant B-lymphocytes diffusely efface the normal architecture of the lymph node or extra-nodal site. The malignant cells are large, transformed lymphoid cells with nuclei at least twice the size of a small lymphocyte. DLBCLs typically express pan-B-cell markers CD19, CD20, CD22, CD79a and often have surface immunoglobulin (50%-75% of cases). CD30 expression may occur, most commonly with the anaplastic variant (ten Berge, Oudejans et al. 2003).

The Bcl-2 protein is expressed in 30-50% of DLBCL, and the prognostic significance of Bcl-2 protein expression is controversial although in most studies it is associated with reduced disease-free survival (Iqbal, Neppalli et al. 2006). A proportion of cases

express CD5 but are cyclin-D1 negative. Expression of CD138 may be seen but mostly in cases showing plasmablastic morphology but is rarely seen in other cases (Rafaniello Raviele, Pruneri et al. 2009).

DLBCL comprises several morphological varieties immunoblastic, centroblastic, anaplastic and T-cell rich (TCR). TCR is relatively uncommon morphologic variant of DLBCL, representing about 3% of all DLBCLs in recent series (Sehn, Donaldson et al. 2005). The entity was first described as "T-cell-rich B-cell lymphoma" in 1988 (Ramsay, Smith et al. 1988), then the term "histiocyte-rich B-cell lymphoma" was introduced by Delabie et al. describing minimal neoplastic B cells amid a prominent infiltrate of histiocytes and small lymphocytes (Delabie, Vandenberghe et al. 1992). In 2001, the World Health Organization (WHO) formally defined T/HRBCL as a morphologic variant of DLBCL characterized by fewer than 10% large neoplastic B cells surrounded by a prominent inflammatory infiltrate, the majority of which are small polyclonal T cells, with or without the presence of histiocytes (Jaffe ES 2001).

### 1.2.4 B-Cell Biology

The cross talk between normal B-cells ontogeny and their malignant counterparts is indispensable for the understanding of lymphomagenesis (Figure 1-2). B cell maturation process can be divided into four stages. The first stage is a B cell progenitor stage before V (D) J recombination. The second stage is the generation of naïve B cells following V (D) J recombination, but before its encounter with antigen. The third stage occurs in the germinal centre (GC) microenvironment and includes somatic mutation; antigen selection and isotype switch events. The fourth stage is the post GC stage, which corresponds to memory B cells and plasma cells, in which the mutational process had been accomplished leading to the production of high affinity antibodies. Non-dividing plasma cells, that are entirely dedicated to Ig synthesis and secretion, are the final

effectors of humoral immunity. The cells in germinal centres demonstrate an extremely high rate of proliferation and apoptosis (Engel and Hedrick 1988).

The first dangerous hurdle in B-cell differentiation is rearrangement of the immunoglobulin genes of B-cell precursors in the bone marrow to form a B-cell receptor (BCR). This molecular process, V(D)J RECOMBINATION, involves double-stranded DNA breaks that are initiated by recombination-activating genes (RAG1 and RAG2) and resolved by the non-homologous end-joining repair apparatus. Occasionally, these breaks are resolved aberrantly, leading to chromosomal translocations. In lymphomas, chromosomal translocations, created by aberrantly resolved DNA breaks, typically replace the normal regulatory sequence of a gene with heterologous regulatory elements that drive inappropriate gene expression near the breakpoints (Oettinger 1992).

After antigen encounter, naïve B cells follow one of three pathways: they can enter the GC microenvironment, where they interact with T cells, follicular dendritic cells and antigen; they can differentiate into short-lived plasmablasts outside of the GC; or they can enter an unresponsive state known as anergy resulting in the production of memory B-cells. In the GC, two molecular processes remodel DNA — immunoglobulin class switch recombination (CSR) and immunoglobulin somatic hypermutation (MacLennan 1994).

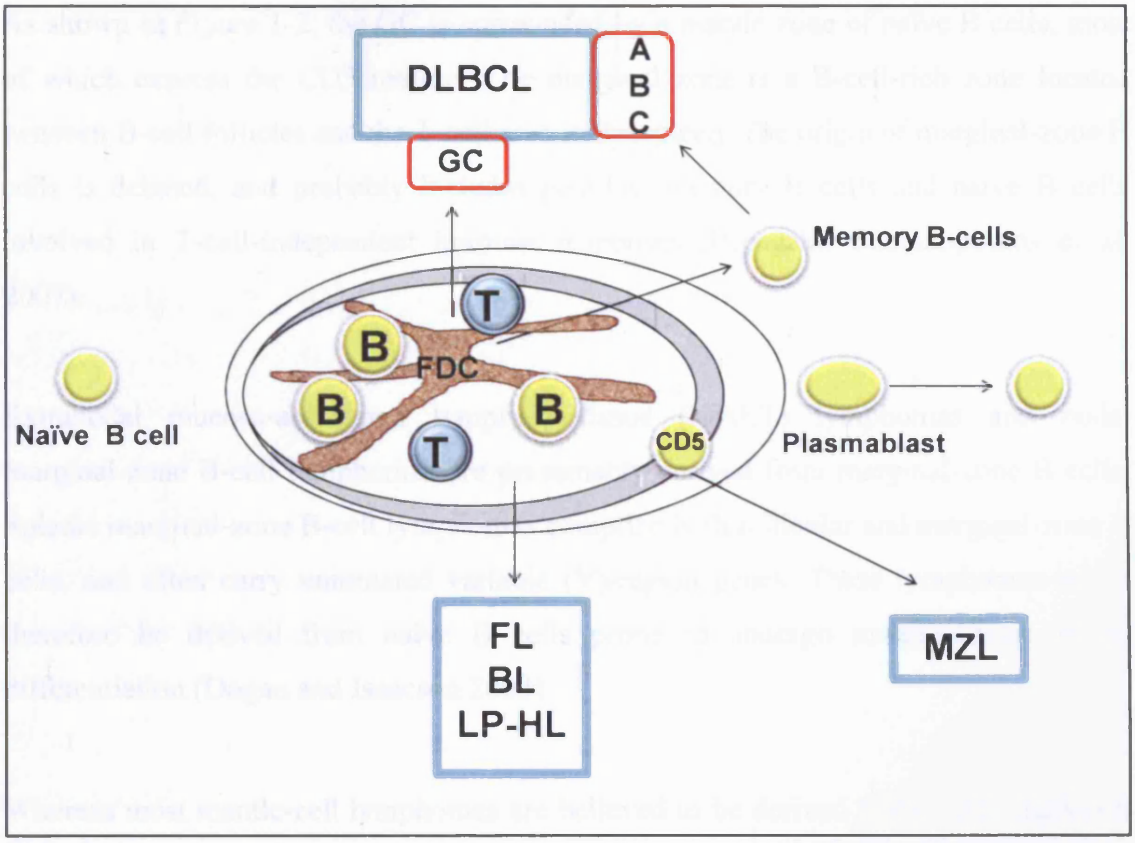



**Figure 1-2: Cell of origin of lymphoma in relation to normal differentiation.** GC B-cell is the origin of Follicular lymphoma (FL), Burkitt's lymphoma (BL), Lymphocyte predominance–HL and GC-DLBCL. Activated B-cell –DLBCL arise from memory B-cells and Mantle zone Lymphoma arise from CD5 B-cells in the mantle zone of the GC. Modified from (Kuppers, Klein et al. 1999).

B-cell lymphomas have been shown to arise from all steps along the B-cell differentiation pathway. Although B-cell lymphomas show features of their nonmalignant counterparts, they also acquire multiple genetic abnormalities that involve their genesis and progression. Gene expression profiling studies have shown that B-cell lymphomas retain at least some characteristics of their cell of origin (Rosenwald A 2003).

Most lymphomas are derived from germinal-centre (GC) B cells or from B cells that have passed through the GC, indicating its role in the pathogenesis of B-cell lymphoma.

As shown in Figure 1-2, the GC is surrounded by a mantle zone of naïve B cells, most of which express the CD5 marker. The marginal zone is a B-cell-rich zone located between B-cell follicles and the T-cell area in the spleen. The origin of marginal-zone B cells is debated, and probably includes post-GC memory B cells and naïve B cells involved in T-cell-independent immune responses (Papadaki, Stamatopoulos et al. 2007).

Extranodal mucosa-associated lymphoid tissue (MALT) lymphomas and nodal marginal-zone B-cell lymphomas are presumably derived from marginal-zone B cells. Splenic marginal-zone B-cell lymphomas comprise both follicular and marginal-zone B cells, and often carry unmutated variable (V)-region genes. These lymphomas might therefore be derived from naïve B cells prone to undergo marginal-zone B-cell differentiation (Dogan and Isaacson 2003).

Whereas most mantle-cell lymphomas are believed to be derived from $CD5^+$ (naïve) B cells of the mantle zone, about 20–30% of cases carry mutated V-region genes, indicating that they have passed through the GC. The origin of B-cell chronic lymphocytic leukaemia (B-CLL) cells has been controversial. About half of the cases of B-CLL carry mutations in V-region genes. Both subsets of B-CLL have been proposed to derive either from $CD5^+$ B cells, memory B cells or marginal-zone B cells (Chiorazzi and Ferrarini 2003).

### 1.2.5 Bcl-6

The proto-oncogene Bcl-6 was identified by virtue of its involvement in chromosomal translocations associated with B cell–derived non-Hodgkin lymphoma (B-NHL). The product of BCL6 is a nuclear phosphoprotein belonging to the BTB/POZ (bric-à-brac, tramtrack, broad complex/Pox virus zinc fingers) zinc finger family of transcription factors. BCL6 can repress transcription from promoters containing its DNA binding site

(Davies, Hawe et al. 1999). In the B-cell lineage, Bcl-6 is selectively expressed in mature B cells within germinal centre (GC), where B cells undergo immunoglobulin gene hypermutation and isotype switching and are selected based on affinity maturation (Oksana et al 2002).

The function of BCL6 is to repress genes involved in the control of lymphocyte activation, differentiation and apoptosis within the GC. Downregulation of BCL6 is necessary for normal B cells to exit the germinal centre, whereas BCL6 remains constitutively expressed in a substantial fraction of B-cell lymphomas. Given the role of BCL6 in DLBCL pathogenesis, BCL6 could be an excellent therapeutic target. However, BCL6 is a fairly ubiquitous protein, and its functions in other organs must also be considered (Parekh, Prive et al. 2008).

Bcl-6 can directly repress the transcription of the TP53 tumour suppressor gene. This allows normal GC and DLBCL B-cells to escape apoptosis in response to DNA damage and allows continued tumour-cell growth (Phan RT and Dalla-Favera R 2004). Recently, BCL6 had been proven as a new target for TP53, regulated via a response element frequently disrupted in B-NHL (Margalit et al 2006).

### 1.2.6 Bcl-2

Bcl-2 (B-cell leukaemia, lymphoma 2) was one of the first apoptosis regulating genes to be identified in cases of follicular lymphomas with t(14;18). The dysregulated expression of Bcl-2 is implicated in may cancers. Over-expression of Bcl-2 in cancer cells leads to resistance to apoptosis induced by anti-cancer drugs and many other apoptotic stimuli (Vaux et al 1988).

Bcl-2 family proteins share regions of conserved sequence similarity termed as BH domains (Bcl-2 homology domains) with all members sharing at least one of the four

identified BH domains (BH1–4) (Frenzel, Grespi et al. 2009). The Bcl-2 family of proteins can be divided into three subfamilies based on their structural and functional homology. The prosurvival (antiapoptotic) subfamily that includes Bcl-2, Bcl-xL and Bcl-w, the second proapoptotic Bax subfamily including, Bax, Bak and Bok and the third proapoptotic subfamily, which includes proteins such as Bid, Bad and Bim. In general, the ratio of antiapoptotic to proapoptotic members is thought to dictate the fate of the cell (Oltvai et al 1993).

*BCL2* is important in normal B-cell development and differentiation (Veis et al 1993). Translocation (14; 18) (q32; q21), which is detected in 20-30% of diffuse large B-cell lymphomas (DLBCL), is regarded as a major mechanism for Bcl-2 protein overexpression. However, increased expression of BCL2 may also result from BCL2 gene amplification in DLBCL. The association of BCL2 protein over-expression with survival of DLBCL is controversial, although a correlation with decreased overall survival seems to exist (Colomo et al 2003).

## *1.3 Tumour Protein 53 (TP53)*

### 1.3.1 Introduction

TP53 is a nuclear phosphoprotein that was discovered in 1979, forming complexes with the large T antigen, in SV40 oncogene transformed cells (Linzer, Maltzman et al. 1979). From this start as a tumour antigen, TP53 stands today as the key tumour suppressor in humans with over 52,000 papers published on this protein, to date. Research on TP53 is now proceeding at a blistering pace, and a complicated picture in which the protein provides exquisite cellular protection coupled with remarkable vulnerability has emerged. Human germ line mutations of *TP53* are responsible for the Li–Fraumeni syndrome (LFS), characterized by elevated risk of developing childhood and early-onset adult malignancies (Li 1990). Transgenic mice deficient in *TP53* are

fertile, undergo normal development immunologically competency, but have a very high incidence of tumours (Donehower, Harvey et al. 1992). In vitro, WT-p53 can suppress or inhibit malignant transformation of cultured cells by viral or cellular oncogenes, and stop growth of pre-transformed cells at the G1 phase of the cell cycle. When DNA is damaged in vivo, TP53 levels increase and cell growth is interrupted, pausing for either DNA repair followed by resumption of normal cell growth or induction of programmed cell death (apoptosis). Cells lacking both functional alleles of *TP53* are unable to respond appropriately to DNA damage, permitting accumulation of mutations and chromosomal rearrangements, and rapid selection of malignant clones (Cadwell and Zambetti 2001).

*TP53* is often referred to as the 'cellular gatekeeper' or the 'guardian of the genome' and its importance is emphasized by the discovery of mutations of *TP53* in over 50% of all human tumours. TP53 functions faithfully by responding to constant bombardment with genotoxic stress; whether causing cell cycle arrest, apoptosis, cellular senescence or differentiation. In many cancers where *TP53* is wild-type, the TP53 pathway may be altered by other oncogenic events. Functional inactivation of TP53 is not always the result of a *TP53* genetic defect. Viral and cellular oncoproteins that are capable of transforming cells in culture have been shown to bind and inactivate TP53 (Hirose, Masaki et al. 2003).

### 1.3.2 TP53 Protein Structure

The *TP53* gene, located at 17p13, encodes a 53kD protein. *TP53* contains 11 exons spanning 20 kb. It belongs to a family of highly conserved genes that also includes *TP63* and *TP73*, encoding p63 and p73 respectively (Benard, Douc-Rasy et al. 2003). The human TP53 protein comprises several domains (Figure 1-3): An amino-terminus part (1-44) contains the transactivation domain, which is responsible for activating downstream target genes; a proline-rich domain (58-101) that mediates TP53 response

to DNA damage through apoptosis. DNA-binding domain (102-292) is a core domain which is the target of most of TP53 mutations found in human cancers, oligomerization domain (325-356), three putative nuclear localization signals (NLS) have been identified in the C-terminus and two putative nuclear export signals (NES) (Berkson, Hollick et al. 2005).

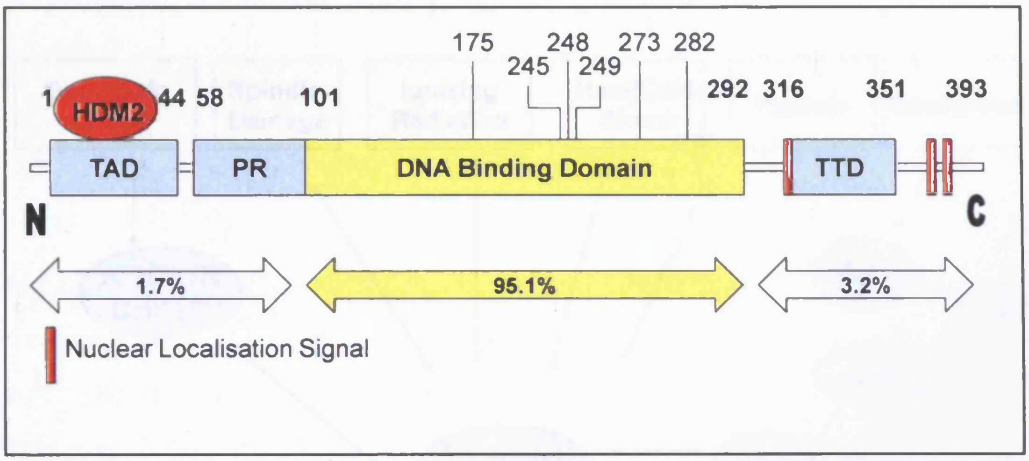



**Figure 1-3: TP53 protein domains.** TP53 contains four highly conserved domains; Transactivation domain (TAD), Proline rich (PR), DNA binding domain and Tetramerisation domain (TTD). Codons 175, 245, 248, 249, 272 and 282 are considered mutation hot-spots. Almost all of the point mutations that are found in cancers occur within the central DNA-binding core of TP53; the percentage of mutations within each region detected in cancers to date is indicated below. Modified from (Berkson, Hollick et al. 2005).

## 1.3.3 TP53 Pathway

### *1.3.3.1 Upstream regulators of TP53 pathway (Input signals):*

TP53 acts as a cellular stress sensor that is activated in response to DNA damage, hypoxia, nucleotide depletion, aberrant growth signals, and chemotherapeutic drugs. All these signals, regardless of the different input signal and the different consequences they

pursue, converge to TP53 as the central node to monitor and respond to cellular stress as shown in Figure 1-4 (Harris and Levine 2005).

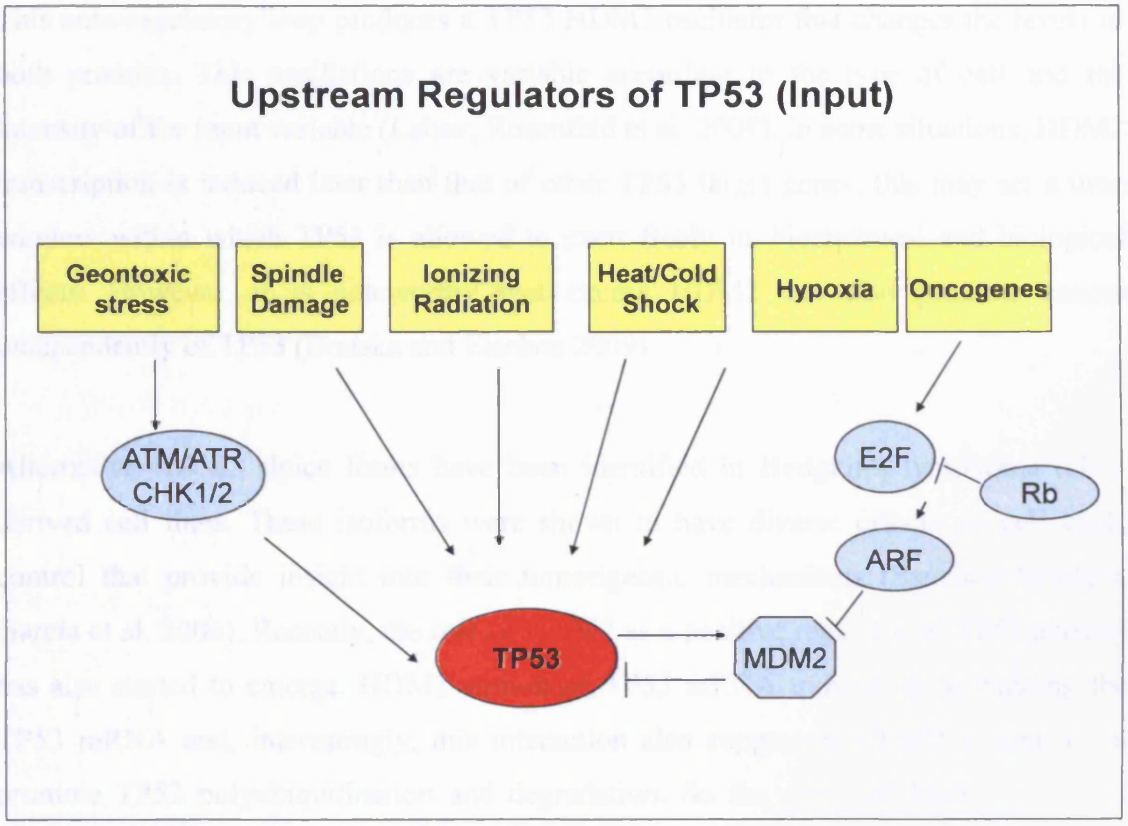



**Figure 1-4: Input signals of TP53.** TP53 senses the DNA damage induced by several input such as oncogenes and genotoxic stress. Modified from (Levine, Hu et al. 2006).

### *1.3.3.2 Core regulation of TP53: The HDM2 negative feedback loop:*

One of the central components of TP53 regulation is the HDM2, the human orthologue of MDM2 (Murine Double Minute Clone 2). The HDM2 gene on chromosome 12q 12-13 is induced by TP53. It encodes a protein that interacts with the N-terminal region of TP53 directly masking the transcriptional activation domain and inhibiting TP53 tumour suppressor functions. HDM2, acting as an ubiquitin ligase, it transfers monoubiquitin tags to the lysine residues of TP53 leading to its degradation. Thus

creating a negative feedback loop in which the TP53 induces the expression of its own negative regulator (Cheah and Looi 2001).

This auto-regulatory loop produces a TP53-HDM2 oscillator that changes the levels of both proteins. This oscillations are variable according to the type of cell and the intensity of the input variable (Lahav, Rosenfeld et al. 2004). In some situations, HDM2 transcription is induced later than that of other TP53 target genes; this may set a time window within which TP53 is allowed to exert freely its biochemical and biological effects. However, it is noteworthy that excess HDM2 can also promote cancer independently of TP53 (Bouska and Eischen 2009).

Alternative HDM2 slpice forms have been identified in Hodgkin's lymphoma (HL)-derived cell lines. These isoforms were shown to have diverse effects on cell cycle control that provide insight into their tumorigeneic mechanisms (Sanchez-Aguilera, Garcia et al. 2006). Recently, the role of HDM2 as a positive regulator of TP53 activity has also started to emerge. HDM2 stimulates TP53 mRNA translation by binding the TP53 mRNA and, interestingly, this interaction also suppresses HDM2's capacity to promote TP53 polyubiquitination and degradation. So the effect of HDM2 on TP53 expression is actually a balance between synthesis and degradation (Candeias, Malbert-Colas et al. 2008).

Other HDM2-independent mechanisms affecting TP53 stability had been also reported. The HDM2 family member MdmX is quickly emerging as a key regulator of TP53 as well. Although the exact function of MdmX remains ambiguous, some evidence clearly implicates MdmX as it is upregulated in many tumours expressing WT-p53 (Parant, Reinke et al. 2001). Apart from the HDM2 family members, once activated the TP53 induces the transcription of other ubiquitin ligases such as *COP-1* (COnstitutive Photochromogenic-1) (Corcoran, Huang et al. 2004) and - (p53-induced RING-H2 domain protein) genes (Logan 2006).

### *1.3.3.3 Downstream regulators of TP53 pathway (Output signals):*

TP53 is involved in several different aspects of cell cycle arrest, apoptosis, control of genome integrity, and DNA repair. TP53 as a tetramer, can bind to specific sequences and thus transactivate a group of genes for example, p21/waf1, GADD-45, HDM2, cyclin G, Bax, and IGF-BP3. Several groups have found that active TP53 is sensed differently at different promoters, resulting in differential DNA binding and transactivation On the other hand, TP53 can also inhibit the expression of some genes such as Bcl-2 and topoisomerase IIa (Taylor and Stark 2001). TP53 activation results in effector functions mediated by TP53-regulated genes that can be broadly divided into four categories; apoptosis, cell cycle arrest, DNA repair and inhibition of angiogenesis (Figure 1-5). The most extensively studied TP53 pathways are apoptosis and cell cycle arrest.

Whilst having multiple downstream targets, the main target for TP53-induced cell cycle arrest seems to be the p21/WAF1 gene. p21 has been identified by virtue of its activation by TP53, its association with cyclin/cyclin-dependent kinase (CDK) complexes acting at the $G_1$-S Transition. p21 binds to and inhibits CDK causing hypo-phosphorylation of Rb, thus preventing the release of E2F and blocking the G1-S transition. Furthermore, the p21 protein was shown previously to interact with the proliferating cell nuclear antigen (PCNA), thereby preventing DNA replication (Cayrol, Knibiehler et al. 1998). Abnormalities of any of the downstream components of the TP53 pathway can have a phenotype similar to TP53 inactivation.

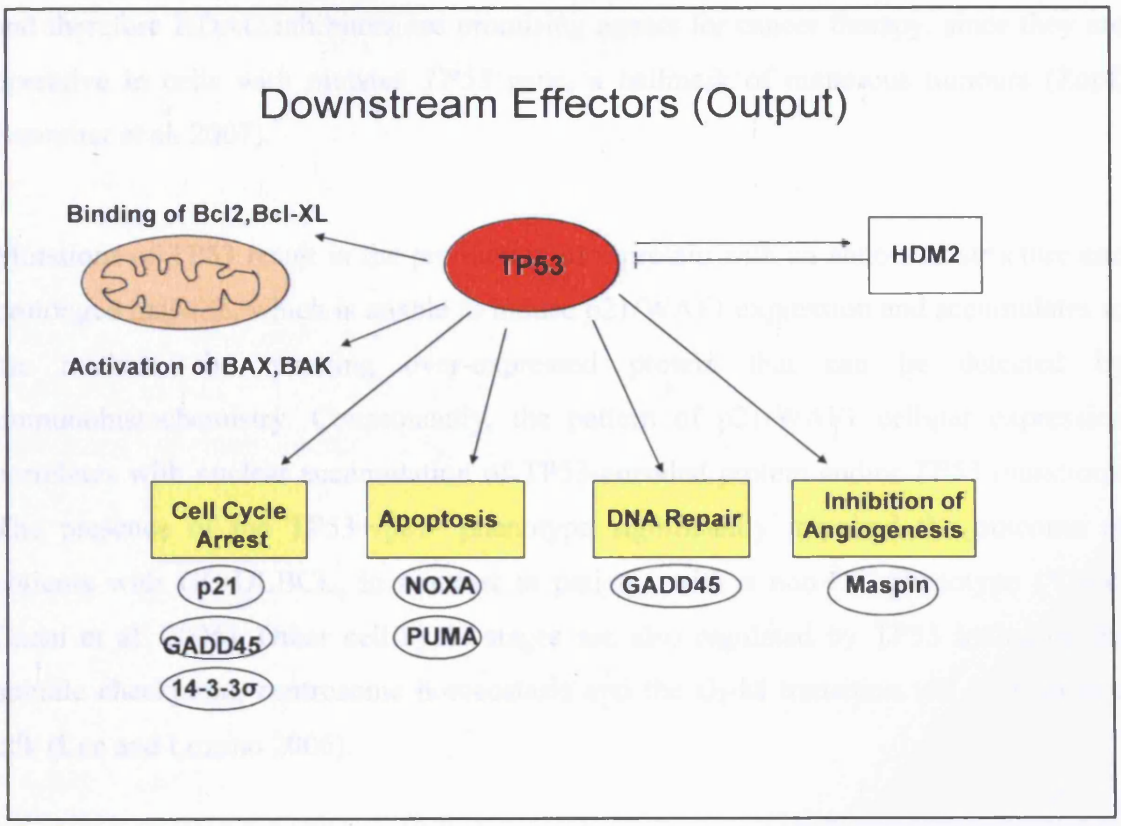



**Figure 1-5: TP53 Output signals.** Several downstream functions are regulated by TP53 such as cell cycle arrest mediated mainly (via p21), apoptosis (via NOXA, and PUMA), DNA repair (via GADD45) and inhibition of angiogenesis (via maspin). Modified from (Caron de Fromentel and Soussi 1992).

Although p21 is activated by TP53-dependent mechanisms in response to DNA damage to ensure cell cycle arrest and repair, a variety of agents that promote differentiation can up-regulate *p21* independently of TP53. Similarly, the p21 gene can be activated by transforming growth factor β, $Ca^{2+}$, lovastatin, or nerve growth factor (Rao, Lowe et al. 1998).

Recently, a number of reports demonstrated the induction of *p21* by inhibitors of histone deacetylases (HDACs). The transcriptional activation of the p21 gene by these inhibitors is promoted by chromatin remodelling, following acetylation of histones H3 and H4 in the p21 promoter region. This activation of *p21* occurs in a TP53-independent fashion,

and therefore HDAC inhibitors are promising agents for cancer therapy, since they are operative in cells with mutated *TP53* gene, a hallmark of numerous tumours (Zopf, Neureiter et al. 2007).

Mutations of TP53 result in the production of a protein with an abnormal structure and prolonged half-life, which is unable to induce p21/WAF1 expression and accumulates in the nucleus, the resulting over-expressed protein that can be detected by immunohistochemistry. Consequently, the pattern of p21/WAF1 cellular expression correlates with nuclear accumulation of TP53-encoded protein and/or *TP53* mutations. The presence of the TP53+/p21- phenotype significantly impaired the outcome of patients with GC-DLBCL, in contrast to patients with a non-GC phenotype (Visco, Canal et al. 2006). Other cell cycle stages are also regulated by TP53 including the spindle checkpoint, centrosome homeostasis and the $G_2$-M transition via 14-3-3δ as a cdk (Lee and Lozano 2006).

### 1.3.4 Regulation of Apoptosis

The process of apoptosis is tightly-checked process that lead to characteristic alterations in cellular and subcellular structure, including cell shrinkage, nuclear fragmentation, and chromatin condensation. The apoptotic pathway is a balance between pro and anti-apoptotic signals. Depending on the extent and the nature of cell stressor, DNA damage can induce cell cycle arrest to give the cell an opportunity to repair damaged DNA or apoptosis, when the damage is beyond repair. Cell cycle arrest induced by TP53 is mainly mediated by the CDK inhibitor (CKI), p21.

Apoptosis mediated by TP53 on the other hand, depends on induction of numerous proteins including pro-apoptotic members belonging to the BH3-only proteins family; Bim (Bcl-2 interacting mediator of cell death), Puma (p53 up-regulated modulator of apoptosis), and Noxa. These proteins instigate the intrinsic mitochondrial death pathway either directly via disruption of the outer mitochondrial membrane, causing the

release of cytochrome-C or smac-diablo, or indirectly by sequestering anti-apoptotic proteins, such as Bcl-2 (Erlacher, Michalak et al. 2005). TP53 also induces the transcription of Bax (Bcl-2 associated X protein) Bak (Bcl-2 homologous killer) which inhibit the main pro-survival protein Bcl-2 as illustrated in Figure 1-6.

Moreover, a transcription-independent TP53-regulated apoptotic pathway was identified, in which TP53 itself localizes to mitochondria and directly regulates the mitochondrial antiapoptotic molecules Bcl-2 and Bcl-xL (Kuribayashi and El-Deiry 2008).

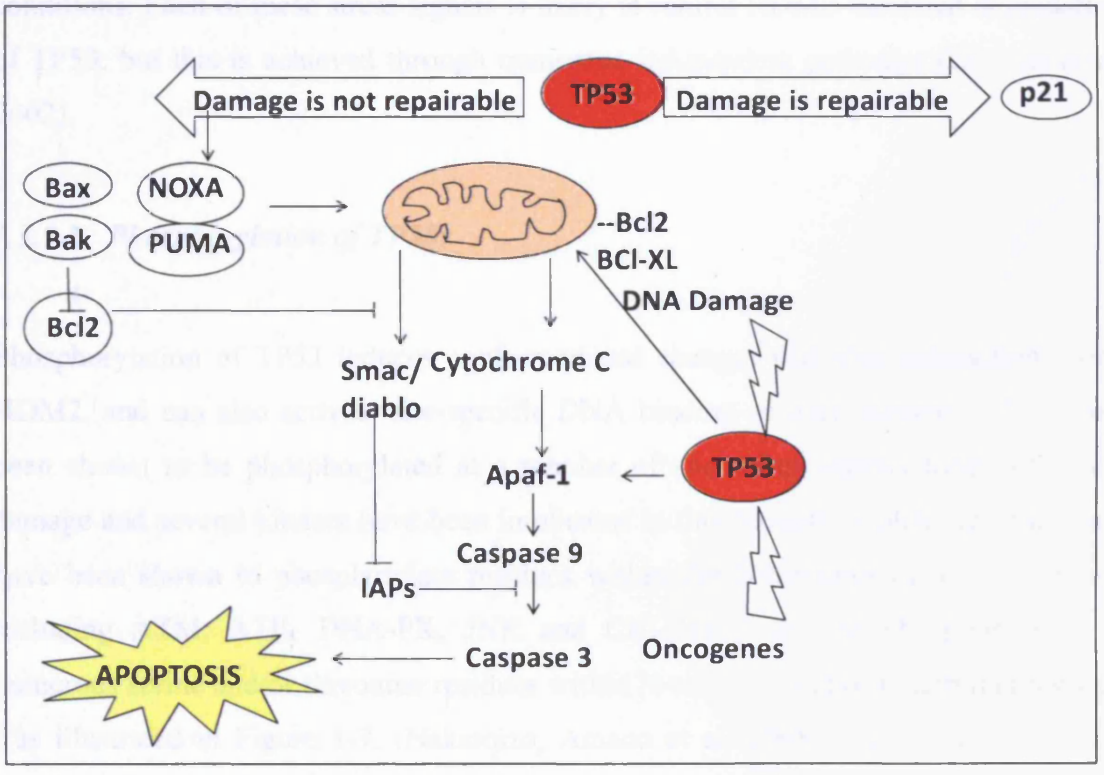



**Figure 1-6 : TP53 regulation of apoptosis.** TP53 induces the proapoptotic proteins PUMA and NOXA and can also directly localise to the mitochondria to regulate the antiapoptotic proteins Bcl-2 and Bcl-XL. Modified from (Agarwal, Taylor et al. 1998).

## 1.3.5 Post-translational modification of TP53

Rapid degradation of TP53 in normal cells is critical to efficiently dampen TP53 activity under normal conditions, while the induction of a TP53 response is intimately related to the stabilization of the TP53 protein. The stabilization of TP53 involves mechanisms to protect TP53 from HDM2 and is a common response to many different and diverse forms of stress. The activation of TP53 and inhibition of cell growth in response to these signals is thought to prevent the development and progression of malignant cells, by preventing accumulation of genomic abnormalities and inhibiting the outgrowth of abnormally proliferating cells, or cells growing under abnormal conditions. Each of these stress signals is likely to inhibit HDM2-mediated degradation of TP53, but this is achieved through numerous independent pathways (Li, Luo et al. 2002).

### *1.3.5.1 Phosphorylation of TP53:*

Phosphorylation of TP53 induces conformational changes that alter interactions with HDM2, and can also activate site-specific DNA binding activity. Recently, TP53 has been shown to be phosphorylated at a number of sites after various forms of DNA damage and several kinases have been implicated in this process. A plethora of kinases have been shown to phosphorylate residues within the N-terminus of TP53 in vitro, including ATM, ATR, DNA-PK, JNK and CKI. TP53 can be phosphorylated at numerous serine and/or threonine residues within N-terminal and/or C-terminal regions , as illustrated in Figure 1-7. (Nakamizo, Amano et al. 2008; Zhao, Traganos et al. 2008; Jenkins, Yamaguchi et al. 2009).

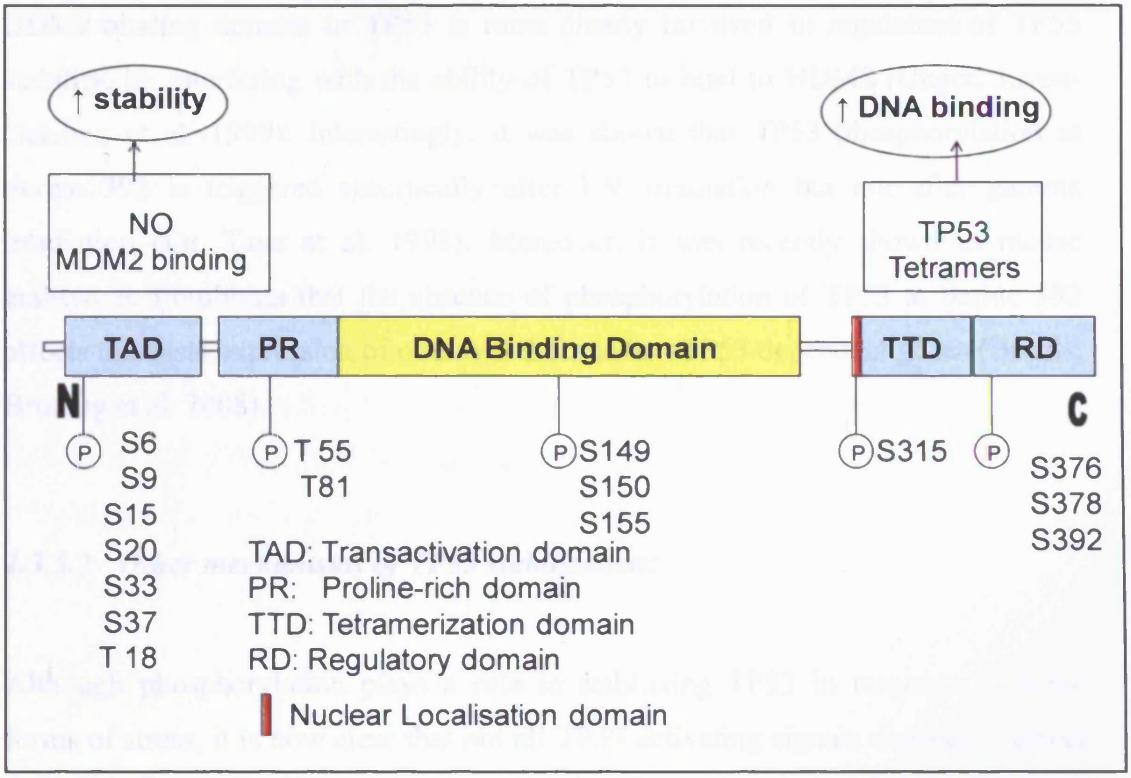



**Figure 1-7: Post-translational modification of TP53 by phosphorylation.** DNA damage triggers TP53 phosphorylation at several N and C terminal residues by different kinases. Modified from (Toledo and Wahl 2006).

On the other hand, HDM2 has been shown to be phosphorylated, possibly leading to an inhibition of its ability to bind TP53, function as an ubiquitin ligase or export TP53 from the nucleus. Although it well documented that TP53 can be phosphorylated at several sites, which may profoundly affect the conformation of TP53, the consequences of such phosphorylation for TP53 function are still unclear (Kulikov, Winter et al. 2006).

The C-terminus of TP53 is of particular interest as a target for induced structural changes. Within its C-terminus, TP53 could be phosphorylated at amino acids 315, 371, 372, 376, 378, 389, and 392, enhancing the in vitro specific DNA-binding activity of TP53 (Qu, Huang et al. 2004). Phosphorylation within the N-terminal

HDM2-binding domain in TP53 is most clearly involved in regulation of TP53 stability, by interfering with the ability of TP53 to bind to HDM2 (Unger, Juven-Gershon et al. 1999). Interestingly, it was shown that TP53 phosphorylation at Serine 392 is triggered specifically after UV irradiation but not after gamma irradiation (Lu, Taya et al. 1998). Moreover, it was recently shown in mouse embryonic fibroblasts that the absence of phosphorylation of TP53 at Serine 392 affects the basal expression of over two thousands – TP53 dependent genes (Bruins, Bruning et al. 2008).

#### *1.3.5.2 Other mechanisms of TP53 stabilization:*

Although phosphorylation plays a role in stabilizing TP53 in response to some forms of stress, it is now clear that not all TP53 activating signals depend on direct phosphorylation. Some activating signals have been shown to specifically inhibit the transcription of HDM2, thereby reducing HDM2 protein levels and increasing TP53 stability The stability of mutant forms of TP53 in cancer cells indicates that loss of TP53 transcriptional activity results in a reduction of HDM2 expression and failure to normally degrade TP53 (Hjerrild, Milne et al. 2001).

Cytoplasmic sequestration of TP53 would result in a similar inability of TP53 to activate HDM2 expression, with consequent stabilization of the TP53 protein. In this case, however, the cytoplasmic TP53 protein would also fail to activate transcription of other target genes that are necessary to mediate cell cycle arrest and apoptosis, and is therefore likely to be defective in activating the full tumour suppressor response. Expression of alternatively spliced versions of HDM2 in tumours has also been correlated with stabilization of TP53 (Jeyaraj, O'Brien et al. 2009).

The best understood of the phosphorylation- independent stabilization mechanisms at the moment involves activation of expression of the tumour suppressor protein $p14^{ARF}$ in humans, $p14^{ARF}$ binds directly to HDM2 in a region distinct from the TP53 binding domain, and inhibits the degradation of TP53 without preventing binding. $p14^{ARF}$ functions both by inhibiting the ubiquitin ligase activity of HDM2 (Honda and Yasuda 1999) and by sequestering HDM2 into the nucleolus, thus preventing nuclear export which is necessary for degradation. The activity of the E2F1, one member of E2F family of transcription factors, is reflected by its ability to stabilize TP53 (Elliott, Dong et al. 2001). Other oncogenes, such as Ras, Myc and E1A have also been shown to stabilize TP53 through $p14^{ARF}$ (Papp, Pemsel et al. 2003).

## 1.3.6 TP53 Family:

### *1.3.6.1 p63/p73*

For a long time, TP53 was assumed to be the only member in his family. This assumption was challenged by the discovery of other TP53 siblings; p63 and p73, in 1997 (Kaghad, Bonnet et al. 1997; Yang, Kaghad et al. 1998). These family members share high level of sequence similarity with TP53 in the DNA- binding domain, allowing them to transactivate TP53-responsive genes causing cell-cycle arrest and apoptosis.

However, although they shared homology, there are striking differences in function between TP53, p73 and p63 as revealed by gene deletion studies. Thus, functional spectrum of the TP53 family therefore exhibits diverse biological effects from development, apoptosis and cell-cycle arrest and oncogenesis family by intermolecular interactions is clearly evidenced by multiple-knockout mice, where TP53 is completely inactive if p63 and p73 are absent (Flores, Tsai et al. 2002)

#### *1.3.6.2 New TP53 isoforms*

Another breakthrough in the field of TP53 research was the discovery of different isoforms. *TP53* gene transcription can be initiated in normal human tissue from two distinct sites upstream of exon 1 and from an internal promoter located in intron 4. Thus, TP53 now has a total of 12 possible mRNAs and 9 proteins (Bourdon, Fernandes et al. 2005). Although it is still in the beginnings, it is clear that the isoforms show different patterns of expression in tissues and cell lines. The expression of some of the isoforms in tumours can infer a possible role in carcinogenesis. Furthermore, the existence of dominant negative isoforms provides an explanation for an impaired TP53 response in the absence of coding region mutations. Moreover, the tissue-specific expression of the TP53 isoforms could explain the tissue-specific regulation of TP53 transcriptional activity in responses to different stresses. Thus, the different output signals adopted by TP53, resulting in the liver cell-cycle arrest in the liver or apoptosis in the spleen in response to the same dose of the same stressor (Marcel and Hainaut 2009).

### 1.3.7 TP53 in DLBCL

The results of several studies support a relation between *TP53* mutations and the development or progression of tumours as well as drug resistance (Sturm, Bosanquet et al. 2003; Henriksson, Baldetorp et al. 2006). Mutation of the *TP53* gene or an accumulation of TP53 protein in tumour cells has been linked to prognosis in several types of cancer. Yet, the prognostic significance of TP53 alterations is still a matter for debate in the case of solid tumours. In B-cell chronic lymphocytic leukaemia, and in multiple myeloma, *TP53* deletions have been associated with shorter survival and poor response to therapy. *TP53* mutation in the cells of aggressive B-cell lymphoma was associated with a poor response to chemotherapy and short survival (Ichikawa, Kinoshita et al. 1997).

Although mutations of *TP53* gene are not a very common event in non-Hodgkin lymphomas (NHL), yet, over-expression of TP53 protein seems to be a frequent finding in these tumours. Mutations of *TP53*, mostly missense, were observed in 20-30 % in DLBCL cases with those in close proximity with DNA associated with a dramatic clinical course (Zainuddin, Berglund et al. 2009).

Cancer-associated mutations in the *TP53* gene occur almost exclusively within the DNA-binding domain (exons 5-8), and inhibit the ability of TP53 to bind DNA in a sequence-specific manner and activate gene transcription. The most frequently observed TP53 alterations occur at *TP53* residues that make direct contact with DNA. Mutations in the DNA-binding domain, including the hot-spot mutations, may also confer a gain-of-function to mutant TP53 that allow them to inhibit WT- p53 or p73, and thus actively contribute to tumour progression. Testing for *TP53* mutations in tumour-derived cells and tissue typically involves DNA sequence analysis of *TP53* exons 5–8, which encode the DNA-binding domain (Lang, Iwakuma et al. 2004).

The impact of TP53 alterations on tumuorigenesis is considerably more than the statistics for *TP53* gene mutation indicate, as WT- p53 may be functionally inactivated by other mechanisms. WT- p53 is normally present at low levels and has a short half-life. In contrast, mutations in *TP53* induce conformational changes which results in altered protein having a longer half-life (4-8 hours) leading to accumulation of mutated protein in cells making it possible to detect by immunohistochemistry (Adamson, Thompson et al. 1995).

## *1.4 Micro RNA 34a as potential TP53 target*

### 1.4.1 Introduction

MicroRNAs (miRNAs) are a recently discovered class of small RNA molecules that play a fundamental role in the regulation of eukaryotic gene expression. They are short 18-24 nucleotide RNAs that function as posttranscriptional gene regulators. These small, non-coding RNAs have diverse functions, including the regulation of cellular differentiation, development, proliferation and apoptosis. It is estimated that vertebrate genomes encode up to 1,000 unique miRNAs, which are predicted to regulate expression of at least 30% of genes (Lewis, Burge et al. 2005). miRNAs- regulated gene expression is based on the study of the first two miRNAs, lin-4 and let-7, in Caenorhabditis elegans. Lin-4 attenuates the translation, but not the mRNA level, of two target genes, lin-14 and lin-28, by imperfect base pairing to complementary sequences in the 3' untranslated region of the target mRNA (Cevec, Thibaudeau et al. 2008).

The central dogma of translational regulation is that gene expression may be controlled by the efficiency of translation of a given mRNA in the absence of a corresponding change in the steady-state level of that mRNA. Translational regulations provide the cell with a more precise and prompt way of controlling expression of proteins that is readily reversible. In addition, translational control also has the advantage of being energy-efficient, that induce rapid changes in protein synthesis without the need for transcriptional activation and subsequent mRNA processing steps (Sen and Roy 2007; Cevec, Thibaudeau et al. 2008).

### 1.4.2 MiRNAs Biogenesis

MiRNAs are generated by a two-step process from long primary miRNAs (pri-miRNAs) that are processed in the nucleus by the microprocessor complex, comprised

of the RNase III enzyme Drosha and the double-stranded RNA binding domain (dsRBD) protein DGCR8 known as Pasha, to 60–70 nt precursor miRNA (pre-miRNA) intermediates (Gregory, Nickels et al. 2004). Hairpin-shaped pre-miRNAs are transported to the cytoplasm by exportin 5, where they are subsequently cleaved by double stranded RNAse Dicer to generate an 22 nt miRNA (Kim 2005).

A single stranded miRNA is incorporated into RNA-induced silencing complex (RISC) the other strand being degraded. RISC/miRNA complex represses protein translation by binding to sequences in the 3' untranslated region of specific mRNAs and results in either endonucleolytic cleavage of targeted mRNA or translational repression depending on the degree of complementarity in the seed sequence, involving base 2-7 of the mature miRNA, between the guide RNA and the miRNA (Bartel 2004; Calin and Croce 2006).

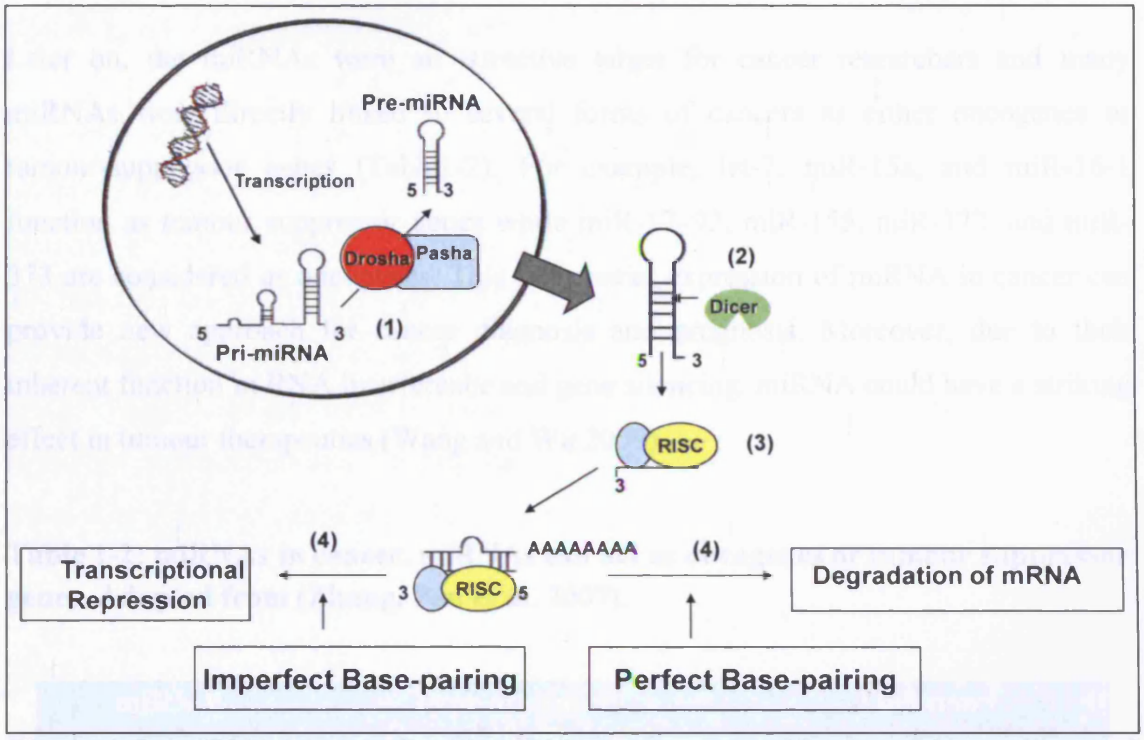



**Figure 1-8: miRNA biogenesis.** (1) miRNAs are expressed in the nucleus as parts of long primary miRNA transcripts (Pri-miRNA) which is digested (Drosha) to produce the precursor miRNA (Pre-miRNA). (2) A cytoplasmic (Dicer) cleaves the pre-miRNA leaving 1–4 nt 3' overhangs. (3) The single-stranded mature miRNA associates with the RNA Induced Silencing Complex (RISC). (4) The miRNA/RISC complex represses protein translation or degrades the mRNA according to base-pairing complementarity. Modified and redrawn from (Saumet and Lecellier 2006).

### 1.4.3 Micro RNA (miRNA) in Cancer

miRNAs have been implicated in the regulation of cell cycle, proliferation and apoptosis, the hall marks of tumorgenesis. Recent evidences indicate that miRNAs can function as tumour suppressors and oncogenes according to their function in cellular transformation and expression in tumours (Cummins and Velculescu 2006). Calin et al. (2002) were the first to show a possible implication of miRNAs in tumourgenesis when they detected miR-15 and miR-16 genes deletion in B-cell chronic lymphocytic leukaemia (CLL) (Calin, Dumitru et al. 2002). In 2005, those miRNA were shown to induce apoptosis by targeting Bcl-2 (Cimmino, Calin et al. 2005).

Later on, the miRNAs were an attractive target for cancer researchers and many miRNAs were directly linked to several forms of cancers as either oncogenes or tumour-suppressor genes (Table1-2). For example, let-7, miR-15a, and miR-16-1 function as tumour suppressor genes while miR-17–92, miR-155, miR-372, and miR-373 are considered as oncogenes. This differential expression of miRNA in cancer can provide new approach for cancer diagnosis and prognosis. Moreover, due to their inherent function in RNA interference and gene silencing, miRNA could have a striking effect in tumour therapeutics (Wang and Wu 2009).

**Table 1-2: miRNAs in cancer. miRNAs can act as oncogenes or tumour suppressor genes. Adapted from (Zhang, Pan et al. 2007).**

| miRNA | Type of cancer | Function |
|---|---|---|
| miR -15a | CLL | Tumour suppressor gene |
| miR -16-1 | CLL | Tumour suppressor gene |
| Let-7 | Lung cancer | Tumour suppressor gene |
| miR-181 | Brain tumours | Tumour suppressor gene |
| miR -17-92 | Lung cancer, lymphoma | Oncogene |
| miR -155 | Breast cancer, leukaemia | Oncogene |
| miR -20a | Lymphoma, lung cancer | Oncogene |
| miR -372, 373 | Testicular germ line tumours | Oncogene |
| miR -21 | Brain tumours | Oncogene |

### 1.4.4 miR-34 family of miRNA

MiR-34s belong to an evolutionarily conserved miRNA family, comprising three members (miR-34a, miR-34b and miR-34c). There are two miR-34 loci in vertebrate genome, one on chromosome 1p36 encoding miR-34a while both miR-34b and miR-34c share a common primary transcript from chromosome 11q23. The expression and consequences of miR-34 activation vary in a tissue-dependent manner. In mice, miR-34a is ubiquitously expressed with the highest expression in brain, whereas miR-34b/c is mainly expressed in lung tissues. Therefore, the two miR-34 genes presumably have tissue-specific functions (Bommer, Gerin et al. 2007).

mir-34a is encoded by the negative strand of chromosome 1(1p36), a region with common loss in many cancers, implicating its tumour suppressor function. Moreover, in cell culture experiments, miR-34 induction contributes to important outcomes of TP53 activation including $G_1$ arrest, cellular senescence and apoptosis. The full spectrum of miR-34 targets is still not fully elucidated, although some key cell cycle and apoptosis effectors have been already validated. These effectors include cyclin E2, E2Fs and Bcl-2 (Lee and Dutta 2006; Petrocca, Visone et al. 2008).

### 1.4.5 TP53 and miRNA:

Mutations of *TP53* occur in more than half of human cancers, this has lead to an extensive research on *TP53* as the most important tumour suppressor gene over the past twenty years. TP53 acts as the guardian of the genome by monitoring cellular stresses and responding accordingly. The effector functions of TP53 as well its upstream signals form a complex network in which *TP53* acts as the main maestro of genomic integrity. TP53 leads to diverse cellular responses varying from cell cycle arrest, activation of apoptosis and DNA damage repair. Which pathway is actually activated, is tightly coordinated in a context- dependent manner. The discovery of extensive network of

miRNAs and their up or down-regulation in cancers could represent another arm in this complex pathway (He, He et al. 2007).

TP53 can act as a link between miRNAs and tumourgenesis by exploiting their RNA interference property. Microarray studies showed that many miRNAs (e.g. (miR-23a, miR-26a, miR-34a, miR-30c, miR-103, miR-107, and miR-18) are regulated by TP53. Among the expressed miRNAs, members of the miR-34 family stand out as direct TP53 targets that presumably mediate different TP53 effector function such as apoptosis and cell cycle arrest. Thus, this family of miRNAs may add new layer to the TP53 complexity (Corney, Flesken-Nikitin et al. 2007).

A genome-wide chromatin immunoprecipitation (ChIP) study by Wei et al noted a presumptive TP53 binding region within 30 kb of the precursor transcription units for both miRNA34a and miRNA34b/c (Wei, Wu et al. 2006). This was followed by other studies involving Immunoprecipitation and luciferase reporters showing that TP53 could bind to and activate transcription of miR-34 (Bommer, Gerin et al. 2007). miR-34 induction contributes to important outcomes of TP53 activation including $G_1$ arrest, cellular senescence and apoptosis.

## *1.5 Rationale, hypothesis and aims of the work*

Diffuse large B-cell lymphoma (DLBCL), the most common type of lymphoma, is an aggressive lymphoma, known to be heterogeneous both regarding morphology and clinical outcome. *TP53* is known to play a critical role in cellular responses since it responds to damaged DNA by induction of G1 arrest, and, if DNA is not repaired, TP53 may induce apoptosis. TP53 dysfunction can induce abnormal cell growth and mutant TP53 can deregulate apoptosis, resulting in increased cell survival, genetic instability and eventually malignant transformation (Sigal and Rotter 2000).

Mutated *TP53* is also associated with resistance to chemotherapy (Harris 1996). Alterations in the *TP53* gene are less common in haematological malignancies than in solid tumours, and in B-cell lymphomas, mutations are found in 10–20% of tumours (Peller and Rotter 2003). Exons 5–8 of *TP53*, which contain highly conserved domains (HCD), have been identified as *TP53* mutational hotspots. Mutations outside hotspot exons account for less than 5% of all mutations and are infrequent in lymphomas (Moller, Ino et al. 1999).

The hypothesis is that although *TP53* mutations are less frequent in lymphomas, TP53 over-expression is quite a common finding. So alterations of TP53 by inactivating mutations, or other mechanisms, could be important factors to disrupt this major tumour suppressor pathway.

The aims of this project were:

- To investigate TP53 expression and functional status by assessing the following proteins which are either regulators or direct targets of TP53. These include p21, HDM2, TP53 phosphorylated at Serine 392.
- To immunophenotypically characterize a cohort of 72 cases of DLBCL using 3 antibodies; Bcl2, Bcl6 and MIB1.

- To identify *TP53* mutations involving exons 5-9 which represent the majority of *TP53* mutations in DLBCL.
- To determine the expression level of miR-34a in DLBCL sections.
- To correlate the immunohistochemical, molecular data of TP53 and related proteins and also with miR-34a.

# Chapter 2

# Materials and Methods

# 2 Materials and Methods:

## *2.1 MATERIALS*

### 2.1.1 Cases Details:

After obtaining the ethical approval for handling human tissue, wax embedded formalin fixed tumour blocks from 72 cases of diffuse large B-Cell lymphoma. The cases were 39 males and 33 female aged 17 to 89 at diagnosis were retrieved from the UHL. Anonymised histopathology reports for were obtained. Validated positive controls from different human carcinomas were used according to the primary antibody of interest. No primary antibody was done in parallel with each run for all antibodies. Normal tonsils fixed in the same way were used as controls for optimization of immunhistochemical analysis.

### 2.1.2 Cell Lines:

Five cell lines were used in the study each harbouring a mutation in TP53 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). They were selected according to the *TP53* mutation status. *TP53* mutations were retrieved according to their *TP53* mutational status from: (http://p53.free.fr/Database/Cancer_cell_lines/p53_cell_lines.html). The individual characteristics for each line and their *TP53* mutations are shown in Tables 2-1 and 2-2.

HCT-15: Epithelial-like derived from colorectal adenocarcinoma. It contains a reported mutation in exon 5.

JURKAT: Round cells derived from human, T -cell leukemia (T-ALL). It contains a mutation in *TP53* exon 6.

RAJI: Lymphoblastoid cells derived from human, Black, Burkitt's lymphoma, and contain a mutation in *TP53* exon 7.

HT29: Epithelial -like cells derived from human, caucasian, colon adenocarcinoma, grade II. It contains a reported mutation in exon 8.

SW-480: Epithelial-like derived from human colon adenocarcinoma Grade III-IV. It contains a reported mutation in exon 9.

Hep-G2: Epithelial-like derived from hepatocellular carcinoma. Wild-type *TP53* cell line which was used as a control in RT-PCR.

**Table 2-1: Cell lines morphology and culture conditions.**

| *Cell Line* | *Morphology* | *Medium* | *Derivation* |
|---|---|---|---|
| HCT-15 | Epithelial-like | 90% RPMI 1640 + 10% FBS | Colorectal adenocarcinoma |
| Jurkat | Round cells | 90% RPMI 1640 + 10% FBS + 2 mM L-glutamine | T -cell leukaemia (T-ALL) |
| Raji | Lymphoblastoid | 90-95% RPMI 1640 + 5-10% FBS | Burkitt's lymphoma |
| HT-29 | Epithelial-like | 90% McCoy's 5A + 10% FBS | Colon adenocarcinoma |
| SW-480 | Epithelial-like | 90-95% RPMI 1640 + 5-10% FBS | Colon adenocarcinoma |

**Table 2-2: *TP53* mutational status in cell lines.**

| *Cell line* | *TP53 mutation* | *Codon* | *Description* | *WT codon* | *Mutant codon* | *WT AA* | *Mutant_ AA* |
|---|---|---|---|---|---|---|---|
| HCT-15 | Exon 5 | 153 | C>G | CCC | GCC | Pro | Ala |
| Jurkat | Exon 6 | 196 | C>T | CGA | TGA | Arg | STOP |
| Raji | exon7 | 234 | T>C | TAC | CAC | Tyr | His |
| HT-29 | exon8 | 273 | G>A | CGT | CAT | Arg | His |
| SW-480 | exon9 | 309 | C>T | CCC | TCC | Pro | Ser |

### 2.1.3 Tissue culture reagents:

| | |
|---|---|
| Dulbecco minimum essential medium (DMEM) | Sigma |
| Dimethyl sulphoxide (DMSO) | Sigma |
| Foetal Bovine serum | Sigma |
| Trypsin/EDTA and sterile phosphate buffered saline | GibcoBRL |
| Flasks (canted neck with 0.2 μm vented plug seal cap) | Falcon |

### 2.1.4 Antibodies and serum

| | |
|---|---|
| Normal rabbit serum | Gibco |
| Strept ABC complex/AP kit | Dako |

Primary Antibodies:

Different antibody dilutions and different buffers in heat induced antigen retrieval were tested on positive controls according to manufactures' data sheet to get the best staining intensity and the least background (Table 2-3).

Secondary antibody: Biotinylated rabbit anti-mouse 1: 400 dilution from Dako.

**Table 2-3: Primary antibodies used in immunohistochemistry.**

| Primary antibody | Company | Clone | Optimised dilution | Buffer | Control tissue |
|---|---|---|---|---|---|
| TP53 | Pharmingen | Do-1 | 1:1000 | citrate buffer pH 6.0 | Colorectal carcinoma |
| p21 | Dako | SX118 | 1:50 | TE buffer pH 9.0 | Gastric carcinoma |
| MDM2 | Abcam | SMP14 | 1:200 | Citrate buffer pH 6.0 | Breast Carcinoma |
| Phospho-p53 | Novocastra | NCL-p53-CMI | 1:200 | TE buffer pH 9.0 | Colorectal carcinoma |
| Ki-67 | Dako | MIB-1 | 1:100 | Citrate buffer pH 6.0 | Normal tonsil |
| Bcl-2 | Dako | 124 | 1:50 | TE buffer pH 9.0 | Normal tonsil |
| Bcl-6 | Dako | PG-B6p | 1:20 | TE buffer pH 9.0 | Normal tonsil |

#### *2.1.4.1 TP53:*

Do-1 mouse monoclonal IgG2a from Dako. It recognizes N-terminal epitope mapping between amino acid residues 11-25 of *TP53* of human origin detecting both WT and mutant TP53. Do-1 was validated to be used on archival histology specimens fixed in formalin and embedded in paraffin (FFPE) (Vogelstein and Kinzler 1992).

#### *2.1.4.2 p21:*

p21 mouse monoclonal antibody from Santa Cruz, clone SX118. The epitope recognized by this antibody is localized to the amino acids 146-164 of p21. It has been validated for immunohistochemical detection of p21 in FFPE sections (Sherr 1993).

#### *2.1.4.3 MDM2:*

SMP14 mouse monoclonal from Abcam. It corresponds to amino acids 154-167 of human MDM2. Its use on FFPE tissue sections has been documented (Zhang and Xiong 2001).

#### *2.1.4.4 Phosphospecific p53:*

NCL-p53-CMI mouse monoclonal antibody clone FP3-2, IgG1. It detects human TP53 phosphorylated at serine 392 and can be readily used on FFPE sections (Matsumoto, Furihata et al. 2004).

#### *2.1.4.5 MIB-1:*

MIB-1 mouse monoclonal IgG1. It has now been established as a reference monoclonal mouse antibody for the demonstration of the Ki-67 which is preferentially expressed during all active phases of the cell cycle (G1, S, G2and M-phases), but it is absent in resting cells (G0-phase). It has been validated for use on FFPE specimens (Huuhtanen, Blomqvist et al. 1999).

#### *2.1.4.6 Bcl-2:*

Clone 124 mouse monoclonal antibody, IgG1. The antibody labels cells that express Bcl-2 oncoprotein and its use on FFPE sections has been validated (Adams and Cory 1998).

#### *2.1.4.7 Bcl-6:*

Mouse Anti-Human BCL6 protein, Clone PG-B6p, is intended for use in immunocytochemistry. The antibody labels cells expressing BCL6 protein and can be applied on FFPE sections (Nakamura 2000).

## 2.1.5 PCR oligonucleotides:

**Table 2-4: Primers used in PCR amplification of TP53 exons 5-9.**

| *Exon* | *Name* | *Sequence* |
|---|---|---|
| ***Exon 5*** | TP53Ex5aF | CTTGTGCCCTGACTTTCAACTCT |
| | TP53ex5aR | CGTCATGTGCTGTGACTGCT |
| | TP53ex5bF | TGTGCAGCTGTGGGTTGATT |
| | TP53ex5bR | AGCAATCAGTGAGGAATCAGAGGCC |
| ***Exon 6*** | TP53ex6aF | CTTGTGCCCTGACTTTCAACTCT |
| | TP53ex6aR | CTTAACCCCTCCTCCCAGAG |
| ***Exon 7*** | TP53ex7aF | TTGGGCCTGTGTTATCTCCTAG |
| | TP53ex7aR | GGGTCAGAGGCAAGCAGAG |
| ***Exon 8*** | TP53ex8aF | CTTGCTTCTCTTTTCCTATCCTGA |
| | TP53ex8aR | TAACTGCACCCTTGGTCTCC |
| ***Exon 9*** | TP53ex9aF | TGCAGTTATGCCTCAGATTCA |
| | TP53ex9aR | AAGAAAACGGCATTTTGAGTG |

## 2.1.6 RT-PCR Analysis:

**Table 2-5: Primers used in RT-PCR expression determination of miRNAs.**

| *Primer name* | *Sequence* |
|---|---|
| 18s Forward | GTAACAAGGTTTCCGTAGGT |
| 18s Reverse | TTGTTGCAACGAACAGGTC |
| MiRNA-34a Forward | TTGGCAATCACTAACTCCACTG |
| MiRNA-34a Stem-loop RTP | GTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAA CC |
| Stem-loop primer | GTCGTATCCAGAATTTGTTGCAACGAACAGGTCTGGATACGACTAA TGATC |
| Common Reverse primer | AGTGCAGGGTCCGAGGTATT |

### 2.1.7 General Laboratory reagents:

| | |
|---|---|
| Thermal cyclers | Perkin Elmer 9700, Gene amp |
| Ammonium Persulphate (APS) | Sigma |
| Boric acid Fischer | Scientific |
| Bromophenol Blue | Sigma |
| Centri-sep spin columns | Princeton |
| dNTPs | Invitrogen |
| EDTA | Sigma |
| Formamide | Sigam |
| Glycerol | Sigma |
| 100 bp ladder | Gibco |
| MDE 2X gel solution | Lonza |
| Magnesium chloride | Sigma |
| β-mercaptoethanol | BDH |
| N,N,N',N'-tetramethylethylenediamine(TEMED) | Sigma |
| QIA quick PCR purificatio Kit | Qiagen |
| Silver Nitrate | Sigma |
| Sodium hydroxide | Sigma |
| Taq DNA polymerase | Promega |
| Tris base | Roche |
| Xylene cyanol | Sigma |

## 2.1.8 Commonly used solutions and buffers:

AJ buffer: 45mM Tris-HCl (pH 8.8), 11mM ammonium sulphate, 4.5 mM magnesium chloride, 110μg/ml BSA, 16.7 mM β-mercaptoethanol, 4.4 mM EDTA (pH8.0), 200 mM dNTPs in water.

Agarose gel loading buffer: 0.2% Bromophenol blue, 0.2% xylene cyanolFF, 0.4 M Tris-acetate, 50% glycerol, 0.02M EDTA, pH 7.5.

Blocking solution: 50ml TBS, 3% BSA, 0.01% Triton X100 and filtered.

Diaminobenzidine solution: 0.5mg/ml Diaminobenzidine, 0.03 hydrogen peroxide.

Eosin Solution: 20g Eosin,1800ml water, 1M1% calcium Chloride, 2Ml 38-40% Formaldehyde.

Ethidium Bromide solution: 10 mg/ml in water

Formamide Dye Mix: 95% Formamide, 0.005% Xylene cyanol FF, 0.005% Bromophenol blue, 10mM EDTA, 0.1% SDS.

Mayer`s Haematoxylin Solution: 0.1% Haematoxylin, 5 ammonium or potassium alum, 0.02% sodium iodide, 0.1 acid, % choral hydrate

Phosphate buffered solution (PBS):137mM sodium chloride, 10mM sodium phosphate, 2.7mM potassium chloride, pH 7.4.

Red Developer: VAB 50 ml, Levamisole 12 mg, FRTR 25 mg and Naphthol 25 mg in 250μl DMF.

Silver Staining of SSCP gels:

- Fixer: 10% IMS, 0.5% Glacial acetic acid
- Stain: 1% silver nitrate
- Developer: 1.5% sodium hydroxide, 0.16% formaldehyde.
- Neutralizer: 0.75% sodium carbonate

10X TBE Buffer: 108g/l Tris, 55g/l boric acid, 5mM EDTA, pH8.0 in water.

SSCP denaturating Buffer: 95% formamide, 0.25 Bromophenal Blue, 0.25 Xylene blue, 10mM sodium hydroxide.

SSCP gel loading buffer: 0.25 Bromophenol blue, 0.25% Xylene Cyanol, 30% Glyceol in water.

Tris-borate-EDTA (TBE): 100mMTris-base, 100mM Boric acid, 2mM EDTA.

Tris buffered Saline (TBS): 137mM sodium chloride, 10mMTris-base, pH7.4.

50X TAE buffer: 0.4 Tris- acetate, 0.2M EDTA, pH 7.5

## *2.2 METHODS:*

### 2.2.1 H&E:

The sections mounted on silanated microscope slides were prepared by immersion in Xylene (5 minutes, twice), followed by graded alcohols (99% 2x1 minute, 9% 1minute). Then they were immersed in Mayer's Haematoxylin solution, for approximately 5 seconds, rinsed in running tap water, briefly immersed in Eosin solution and rinsed in running tap water. The stained sections were then dehydrated by immersion in graded alcohols (95% and 99% IMS for 15 seconds each) and transferred to Xylene before being mounted using DPX mountant.

#### *2.2.2.1 Coating Of the slides:*

Vecta bond was used for coating microscope slides to increase the adherence of the sections to the microscope slides.

### *2.2.2.2 Assessment of TP53 by Immunohistochemistry*

Sections were prepared by immersion in Xylene (5 minutes x2) followed by graded alcohols (99% 2x1 minute, 9% 1minute), washed in running tap water ( 5minutes). Heat induced antigen retrieval was done by microwaving the slides in a 10mM citrate buffer pH 6.0 at 750 watts for 20 minutes. The sections were left to cool slowly to room temperature for at least 45 minutes.

The sections were covered with 100-200µl (according to the size of the section) of 20% normal rabbit serum diluted in blocking solution for 10 min in a humid chamber at room temperature to block non-specific staining. Excess serum was then carefully removed with a tissue and a 1:1000 dilution of primary antibody (DO-1) was added to each section and allowed to incubate in the humid chamber overnight at 4°C. The primary antibody was then washed off using TBS.

Then, 100µl of a 1:400 dilution of biotinylated secondary antibody (rabbit anti-mouse) was added to each section and incubated for 30 min at room temp. During this incubation time, the Avidin –Biotin -Alkaline phosphatase complex was prepared as manufacturers' instructions.

The secondary antibody was washed off and the ABC-AP solution added (100µl-200µl per section) and incubated at room temperature for 30 min. TBS was used to wash off the ABC solution and 200µl of NBT/BCIP were added for 20 minutes. The NBT/BCIP solution was prepared as follows; one tablet of NBT/BCIP is dissolved in 10 ml of UP water, hand shaken, and filtered twice.

The slides were then washed in TBS, followed by, running tap water for 5 min and counter stained briefly for 10 sec with Mayer's Haematoxylin. The sections were finally dehydrated by immersing in graded alcohol to xylene and mounted using aqueous mountant.

Assessment of the TP53 was done by testing 10 different fields of the section and counting and taking photograph of the immunopositive cells in the ten fields and. The number of immunopositive cells was divided by the total number of the counted cells, based on the total count of cells identified by B-cell markers CD20 and CD79a in similar fields. The expression was defined as the percentage of positive cells in the total number of the counted cells.

#### *2.2.2.3 Assessment of p21, MDM2 and phospho-p53 by Immunohistochemistry*

Other Antibodies were stained in the same way with some modifications:

- p21 immunohistochemistry: Heat induced antigen retrieval using TE buffer pH 9.0.
- MDM2 immunohistochemistry: Heat induced antigen retrieval using citrate buffer 6.0. Red developer was used for 60 minutes instead of NBT/BCIP.
  The red developer was prepared in the fume hood by dissolving 12 mg of Levamisole, followed by 25 mg of FRTR in 50 ml of VAB. Finally, mg25 mg of Naphthol was dissolved in 250 µl DMF and added to the solution. The solution was then filtered.
- Phospho- p53 immunohistochemistry: Heat induced antigen retrieval using TE buffer pH 9. Novalink polymer kit replaced ABC-AP complex and DAB solution replaced NBT/BCIP. DAB was prepared by adding 1ml DAB to 50 µl chromogen and was left on sections for 5 minutes. The sections were then rinsed, counterstained and mounted using the DPX mountant.

## 2.2.3 DNA extraction:

### *2.2.3.1 From Fixed tissues*

Areas of interest were first determined by examining the H&E stained section. The Formalin fixed, wax embedded sections were dewaxed and rehydrated through 99% IMS and 95% IMS and left to air dry.

Areas containing the tumour cells were carefully scrapped in 500µl of 0.05 Tris pH 8/0.1% SDS. Proteinase K was then added to give a concentration of 1mg/ml and overnight incubation was done for 48 hours. This incubation was further increased to 4 days to enhance the DNA yield.

- Phenol / chloroform purification:

Equal volume of phenol/24:1; chloroform:isoamyl alcohol (pH 7.5) was added, the solution mixed briefly and then centrifuged at 13,000 rpm for minutes. The aqueous layer containing the DNA was decanted into a fresh eppendorf and the process repeated. The aqueous layer containing the DNA was eluted again into a fresh eppendorf.

- Ethanol precipitation:

To the extracted DNA, 2.5 volumes of 100% Ethanol 1/10 volume of 1M NaCl and 1µl glycogen were added, this was mixed and left at -20°C to precipitate or overnight . The DNA was pelleted at 13,000 rpm for 15 minutes at 4 °C and the supernatant removed. The remaining pellet was washed (with care) with 70% ethanol /IMS and spun for a further 15 minutes at 4 °C. The supernatant was once again removed and the sample was left to air dry for approximately 15 minutes. The pellet was re-suspended to the original volume of sterile UP water (30-50µl). Later on the Qiagen extraction kit was used for extraction of DNA from paraffin embedded sections as this was attributed to higher DNA quality and better SSCP staining of the bands.

#### *2.2.3.2 DNA extraction from cell lines*

Five cell lines were used as positive controls for *TP53* SSCP analysis as they contain reported *TP53* mutations within the exons of interest as previously mentioned in materials section. The cells were grown to approximately 90% confluency at 37 °C in washed with sterile PBS and then harvested by incubation at 37 °C for 2-3 minutes in a trypsin/EDTA solution to encourage detachment from the flask walls. The walls of the flasks were then washed with warm PBS and the cell suspension centrifuged at 1000 rpm for 5 minutes.

The cells were then pelleted (centrifuged at 1000 rpm for 5 minutes), re-suspended in rapid extraction buffer containing Proteinase K and incubated for 3 hours at 56 °C. The DNA was then purified by Phenol: chloroform extraction as previously described.

### 2.2.4 PCR

The DNA, micro-dissected from tumour samples and extracted from cell lines, was amplified using Hot Start PCR on Perkin Elmer thermal cycler. The sequences of the oligonucleotides used are given in materials section. The samples were subjected to 40 cycles of amplification under the following conditions (Table 2-6).

Reaction

| | |
|---|---|
| 10X AJ Buffer | 5µl |
| Template DNA | 1µl |
| Primers (F/R) | 1µl each |
| Taq Polymerase | 1 unit |
| PCR grade water | 40µl |

**Table 2-6: PCR conditions for primer sets used.**

| *Primer* | *Denaturation* | *Cycle conditions* | *Extension* |
|---|---|---|---|
| 5F/5R | 94°C 5 min | 94°C 30 secs; 60°C 30 secs; 72°C 30 secs | 72°C 7 min |
| 6F/5R | 94°C 5 min | 94°C 30 secs; 60°C 30 secs; 72°C 30 secs | 72°C 7 min |
| 7F/5R | 94°C 5 min | 94°C 30 secs; 60°C 30 secs; 72°C 30 secs | 72°C 7 min |
| 8F/5R | 94°C 5 min | 94°C 30 secs; 60°C 30 secs; 72°C 30 secs | 72°C 7 min |
| 9F/5R | 94°C 5 min | 94°C 30 secs; 60°C 30 secs; 72°C 30 secs | 72°C 7 min |

### 2.2.5 SSCP Gel Analysis

Single Stranded conformation polymorphism (SSCP) is a gel electrophoresis based technique, which allows the detection of mutations and polymorphisms in PCR products. First announced in 1989 as a new means of detecting DNA polymorphisms, or sequence variations, SSCP analysis offers an inexpensive, and labour efficient method for scanning for genetic variation before sequencing (Sunnucks et al. 2000).

It is based on the principle that the electrophoretic mobility of a single stranded DNA through a gel is dependent on size, charge and shape of the molecule, unlike the mobility of double stranded DNA which is dependant only on size. Under non-denaturing conditions, single stranded DNA has a folded secondary structure that is determined by intramolecular interactions dictated by its nucleotide sequence.

A single nucleotide difference between two similar sequences is sufficient to alter the secondary structure of one relative to the other, resulting in the difference in mobility of the sequences. Under specific conditions, this altered mobility can be detected visually on the gel, and the resulting band shift sequenced to investigate the type of alteration present as shown in Figure 2-1.

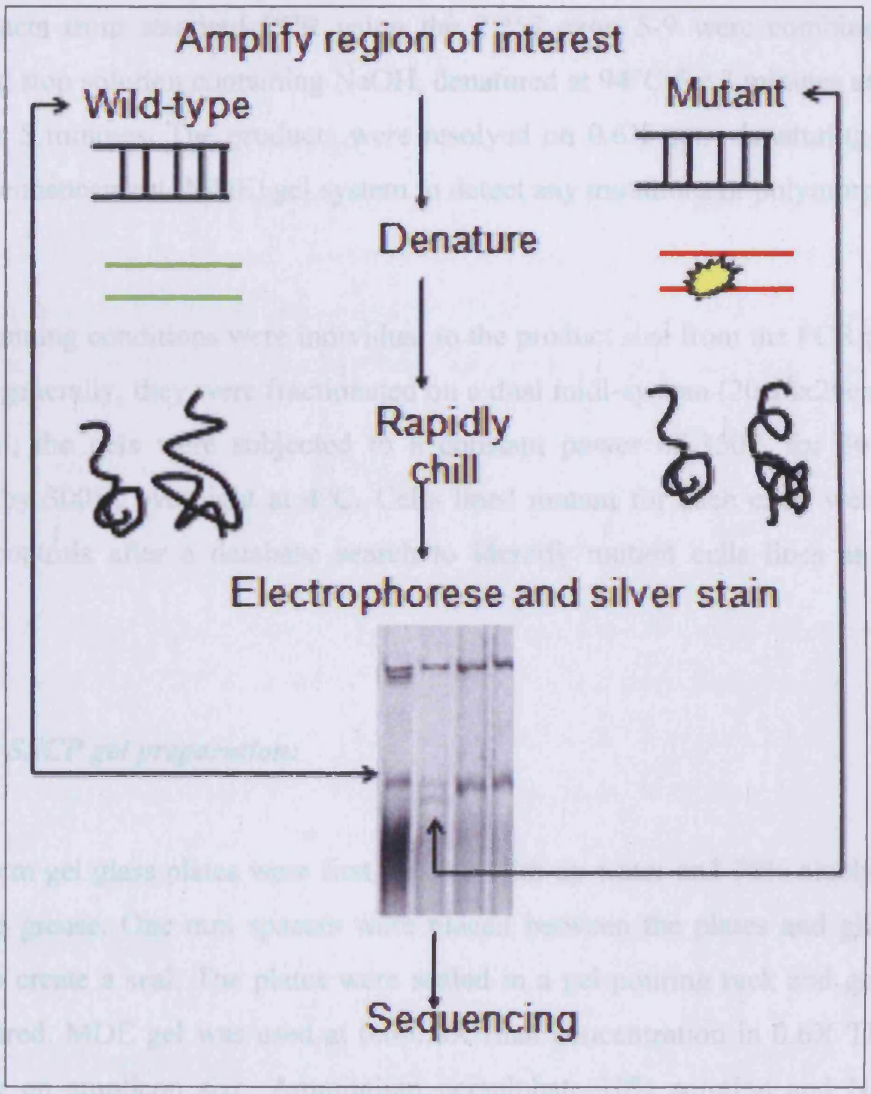



**Figure 2-1: The principle of SSCP analysis.** Modified from (Gasser, Hu et al. 2006). The DNA of interest is subjected to PCR amplification, followed by denaturation at 94°C for 3 minutes followed by rapid chilling on ice. The products are then resolved on mutation detection enhancement (MDE) gel system. Aberrant bands are selected for further sequencing.

The cases were screened for mutations and/or polymorphisms using SSCP analysis following PCR amplification performed on tumour samples using oligonucleotides designed from the flanking intronic sequences of 5 individual exons of the *TP53* gene (exons 5-9 as described in materials).

The products from standard PCR using the *TP53* exon 5-9 were combined with a denaturing stop solution containing NaOH, denatured at 94°C for 3 minutes and chilled on ice for 5 minutes. The products were resolved on 0.6X non- denaturing mutation detection enhancement (MDE) gel system to detect any mutations or polymorphisms.

The gel running conditions were individual to the product size from the PCR primer set used, but generally, they were fractionated on a dual midi-system (20cmx20cm) at 4°C. In general, the gels were subjected to a constant power of 350v, for 30 minutes, followed by 300V, overnight at 4°C. Cells lined mutant for each exon were used as positive controls after a database search to identify mutant cells lines as stated in materials.

#### *2.2.5.1 SSCP gel preparation:*

A 20x20 cm gel glass plates were first washed with up water and 75% alcohol 5 times to remove grease. One mm spacers were placed between the plates and glisseal was applied to create a seal. The plates were sealed in a gel-pouring rack and gel solution was prepared. MDE gel was used at 0.6-0.8X final concentration in 0.6X TBE buffer depending on amplicon size. Ammonium persulphate 10% solution and N,N,N`,N`-tetramethylethyl enediamine (TEMED) were added to final concentration of 0.5µl/ml. The gel was poured between the plates using a syringe and 1mm comb was inserted. The gel was then allowed to set at room temperature for 2 hours.

#### *2.2.5.2 SSCP sample preparation:*

Adjusted volumes (3-8.5µl) of PCR products, according to band intensity on 3% agarose gel, was added to 9µl SSCP loading buffer and heated to 95°C for 5 minutes and immediately chilled on ice for 5 minutes. The PCR product/SSCP loading buffer was then loaded onto the gel and the gels were run at 500 V for 30 minutes followed by 270-350 V overnight depending on amplicon size.

#### *2.2.5.3 Silver staining*

The gels were first fixed by incubation in 10% IMS/0.5 Acetic acid for 2x3 minutes followed by a 0.1% solution of Silver Nitrate for 15 minutes. After rinsing briefly in 2 changes of water, the gels were developed in an aqueous solution of 1.5% NaOH/16% formaldehyde until the bands became clear. This was then discarded and replaced by 0.05 Na2Co3 as the neutraliser for at least 1 hour then the gels were photographed.

#### *2.2.5.4 Selection of Aberrant bands:*

A sterile, disposable needle was used to stab the gel over the band. Then, this needle was then placed into 20µl of standard PCR buffer in a thin-walled eppendorf and then discarded, and the scratched products re-amplified with 40 cycles of PCR using the same primer set. The re-amplified products were run on a 3% agarose gel.

### 2.2.6 Sequencing:

#### *2.2.6.1 DNA purification*

The successfully re-amplified products were subjected to a clean-up procedure using the QIAquick DNA Cleanup System (Qiagen). This purification system was used to remove PCR components such as primers and enzymes as well as traces of MDE gel in the product. This was achieved efficiently using a bind-wash elute procedure to remove possible contaminants.

#### *2.2.6.2 Sequencing Reaction*

The automated service offered by the Protein and Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester, uses the ABI prism377 sequencer. Completed and

purified reactions were provided to them for sequencing. The sequencing reaction was performed using the Big Dye Terminator protocol.

The purified PCR products (3-8.5µl according to the strength of bands) were added to 8µl Big Dye Terminator Mix and 3.5µl PCR primer. The reaction mix was subjected to 40 cycles of PCR as follows:

| 96°C: 10 seconds; 50°C: 5 seconds; 60°C: 4 minutes; soak at 4°C |
|---|

The samples were then purified again using the Centri-Sep columns to remove un-incorporated dyes and primers. The sequencing data was received electronically and subsequently analysed using the Chromas Software 1.45 and 2. The sequence copied in was compared to wild-type *TP53* sequence using the Blast-n search: (http://blast.ncbi.nlm.nih.gov/Blast.cgi).RNA extraction and production of cDNA using stem loop RT primer.

Cell line lysis in Tri-Reagent™ (Sigma) was thawed out as required. Total RNA was then extracted using Tri-Reagent™ as described by the manufacturers. Tissues are micro-dissected under the guidance of H&E stain of the same tumour section. The RNA pellet was then air dried before being re-suspended in sterile ultra-pure water. Paraffin sections from normal tissue and DLBCL cases, not belonging to the T-cell rich subtype, were extracted using 0.05M Tris pH 8.0. 0.1% SDS before being incubated overnight at 37°C with proteinase K (final concentration of 0.5 µg/ml).

Total RNA was then extracted using Tri-Reagent™ as described in the manufacturer's instructions. The total RNA was then used to produce cDNA using AMV reverse transcriptase (3.33 U/µl) (Promega), 1X RT buffer (Promega), 0.25mM dNTPs, 0.25 U/µl RNase inhibitor and 55 nM stem-loop primers (Sigma) as described in. The 25µl

reactions were then incubated in a thermal cycler (7500 fast Applied Biosystems) for 30 mins at 16 °C, 30 mins at 42 °C, 5 mins at 85 °C followed by soak at 4 °C.( Chen at al 2005). The cDNA was then stored at 4 °C until required for RT-PCR. Appropriate RT reactions were also carried out using sterile ultra-pure water.

### 2.2.7 Real Time PCR detection of miRNA expression:

Real time PCR was carried out using a SYBR™ green PCR protocol on a Step-one Plus™ PCR machine The 10µl PCR reaction contained 4 µl cDNA (diluted 1:5), 0.2µl from forward and reverse primer (2nM), 5µl SYBR Green Master mix™ and 0.06µl water. The reactions were incubated in a 96-well plate at 95 °C for 10 min, followed by 95 °C for 15 seconds and 62 °C for 1 minute. The reactions were run in triplicate with appropriate RT reactions and water blank also being run. The cycle threshold (Ct) was then determined using the default threshold settings.

The mature miRNA nucleotide sequences were obtained from the miRBase at the Sanger institute (http://microrna.sanger.ac.uk/) .The miRNA primers were designed using Primer 3. Stemloop primer for miR- 34a was designed by Dr. James H Pringle, Department of Cancer studies and Molecular Medicine, University of Leicester. All of the oligonucleatides used for PCR analysis were validated by performing a BLAST (Basic Local Alignment Search Tool) search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Housekeeping Genes 18S rRNA was used as the endogenous control for normalization in RT Quantitative analysis of gene expression.

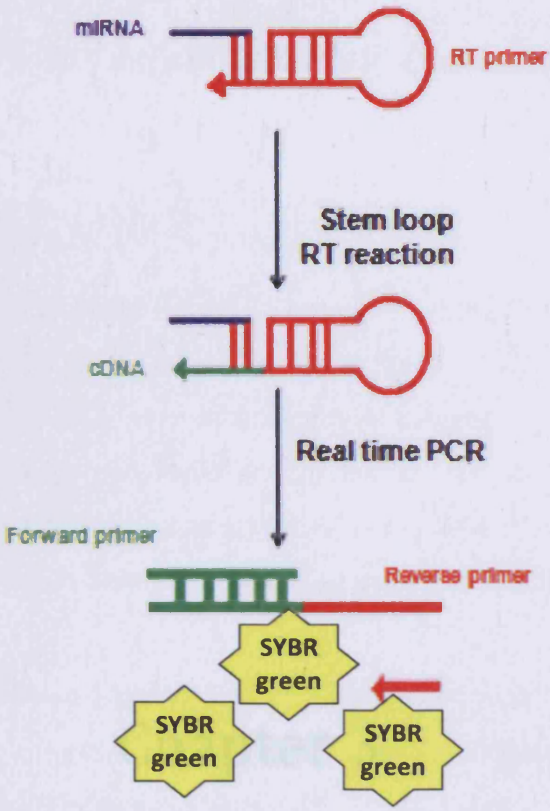



**Figure 2-2: The principle of Stem loop RT reaction** (Modified and redrawn from Chen at al 2005).

## 2.2.8 Statistical analysis:

The data variables were grouped as scale, nominal or ordinal. Two-tailed p values were generated for two contingency tables, using Fisher's exact test, and for larger tables using Chi-square analysis. Spearman's correlation test was used for assessment of correlation between two continuous variables. Non-parametric Mann-Whitney Test was used to compare the median age of diagnosis between different phenotypes. Kruskall-Wallis test was used to correlate the proliferation index and other parameters. The program SPSS for Windows Release 10 was used for statistical analysis. The results were considered as statistically significant when $p<0.05$.

# Chapter 3

# Immunohistochemical analysis of TP53 pathway

# 3 Chapter 3: Immunohistochemical analysis of TP53 pathways

## *3.1 Introduction:*

Diffuse Large B-cell lymphomas (DLBCL) represent the most common type of NHL in Western world. They are characterised by heterogeneous clinical, immunophenotypic and genetic features. DLBCL is a heterogeneous category comprising all lymphoid tumours composed of large neoplastic B cells that do not satisfy the criteria of other more accurately delineated lymphoma entities (Harris, Jaffe et al. 2000). Alterations of several cell cycle regulators have been identified in DLBCL including the *TP53* gene.

Activation of TP53 induces a plethora of effector functions. It was estimated to induce or repress more than 150 genes involved in cell cycle regulation (Vousden 2000). The p21/WAF1 gene is a transcriptional target of TP53, required for cell cycle arrest in response to DNA damage and cellular stress, via its function as a cyclin-dependent kinase inhibitor. Because the p21/WAF1 gene itself is not a target for mutations, identification of the p21-negative phenotype most likely reflects the presence of a non-functional, mutant TP53 that has been also associated with an aggressive clinical course (Shariat et al 2003).

The HDM2 proto-oncogene binds to TP53 and acts as a negative regulator, inhibiting its transcriptional transactivation activity Over-expression of HDM2 may overcome WT p53-mediated suppression of transformed cell growth, and it is one of the mechanisms inactivating TP53 function. Because TP53 transactivates HDM2, over-expression of HDM2 products in the context of an altered TP53 could be explained by mutations acquiring "gain of function". Such paradigm for *TP53* mutations has been reported to enhance tumorigenicity, metastatic potential, and resistance to certain therapeutic agents (Foroutan, Ali Ruf et al. 2007). Alternatively, HDM2 over-expression could be produced by the transactivating functions of the recently described TP53 homologues, p63 and p73 (Kato, Shimada et al. 1999).

Post-transcriptional modification of TP53 by phosphorylation of numerous serine and threonine residues within the N- and C-terminal regions of this protein has been proposed to be an important mechanism by which TP53 stabilization and function are regulated (Meek 1994). Phosphorylation of TP53 at Serine 392 was the more frequent modification among other possible phosphorylation sites of TP53 in human tumour-derived cell lines and tumour tissues (Minamoto, Buschmann et al. 2001). Moreover, Serine 392 phosphorylation regulates the oncogenic function of mutant *TP53* by facilitating cellular resistance to DNA-damaging agents (Yap, Hsieh et al. 2004).

## *3.2 Aims:*

- To investigate TP53 and related protein expressions in DLBCL.

## *3.3 Objectives:*

- To investigate TP53 expression and functional status by assessing the following proteins which are either regulators or direct targets of TP53 namely, p21, HDM2, TP53 phosphorylated at Serine 392.
- To characterize by immunophenotyping a cohort of 72 cases of DLBCL using 3 antibodies; Bcl2, Bcl6 and MIB1.
- To correlate the results of the routine immunohistochemical panel used for DLBCL diagnosis with TP53 and related protein expressions.

## *3.4 Results:*

### 3.4.1 Optimisation of Immunohistochemical analysis:

Formalin fixed, paraffin embedded (FFPE) tumour sections from 72 cases of DLBCL were immunohistochemically assessed for TP53, p21, HDM2 and phospho-p53, Bcl-6, Bcl-2 and MIB-1. Immunohistochemisry was done as a part of the diagnostic work-up routinely done in the pathology department, UHL.

TP53 immunohistochemical analysis had been previously validated in the lab and hence no further optimisation was required. Generally, all sections were dewaxed and antigen retrieval was carried out for 20 minutes at 750 watts. However, the buffer used in heat-induced antigen retrieval was optimised according to the antibody of interest, as shown in Table 3-1. The primary antibody was incubated overnight at 4°C. ABC-AP, from Dako was used and different chromogens were tested for each antibody. The red developer was used in all antibodies except for TP53 and p21, where NBT/BCIP was used instead. The final parameters were selected based on optimal staining on positive control tissue coupled with the minimal background staining (Table 3-1).

**Table 3-1: Different optimisations used for immunohistochemistical analysis.**

| *Primary Antibody* | *Dilutions* | *Antigen Retrieval Buffer* | *Chromogen* | *Positive Control* |
|---|---|---|---|---|
| p21/Waf1 | 1:50*<br>1:100<br>1:200<br>1:500<br>1:1000 | • TE buffer (pH 9.0)*<br>• Citrate buffer (pH 6.0) | • Red developer<br>• NBT/BCIP* | Gastric carcinoma |
| HDM2 | 1:50<br>1:100<br>1:200*<br>1:500<br>1:1000 | • TE buffer (pH 9.0)<br>• Citrate buffer (pH 6.0)* | • Red developer*<br>• NBT/BCIP | Breast carcinoma |
| Phospho-p53 | 1:50<br>1:100<br>1:200*<br>1:500<br>1:1000<br>1:500 | • TE buffer (pH 9.0)*<br>• Citrate buffer (pH 6.0) | • Red developer*<br>• NBT/BCIP | Colorectal carcinoma |

* refers to the dilution, buffer and chromogen adopted in the study.

### 3.4.2 Analysis of immunohistochemical staining:

After proper optimisation of each antibody, the immunohistochemical analysis of TP53, p21, Phopho-p53 and HDM2 was performed for all cases. A positive tumour control and a negative parallel section for each case, where TBS replaced the primary antibody, were included in each run.

The scoring of each immunohistochemical marker was performed in the light of CD20 and Cd79a expression in similar fields. This was done to ensure the expression of the protein of interest was assessed in malignant B-cells only. A total B-cell count was estimated in at least 5 High power fields (HPF), the total number of the cells expressing the immunohistochemical marker was counted and a percentage score was calculated as shown in Table 3-2. The total numbers of B-cells in TCR lymphoma cases ranged from 70-120 cells/HPF and the corresponding number in classic DLBCL ranged from 200-250 cells/HPF.

**Table 3-2: Evaluation of Immunhistochemical analysis for each antibody used.**

| *Antibody* | *Evaluation* | *Reference* |
|---|---|---|
| TP53 | Positive cells where counted in ten HPFs and the percentage was estimated based on the average of counts in the ten fields. Cases were considered as negative if < 20% and positive if ≥ 20%. | Modified from (Walker and Levine 1996) |
| p21 | The number of positive cells was counted in 1000 cells in at least 5 HPFs and the percentage was estimated. Cases were considered as negative if < 5% and positive if ≥ 5%. | (Hinnis, Luckett et al. 2007) |
| HDM2 | The number of positive cells was counted in 1000 cells in at least 5 HPFs and the percentage was estimated. Cases were considered as negative if < 10% and positive if ≥ 10%. | (Moller, Nielsen et al. 2002) |
| Phospho-p53 | The number of positive cells was counted in 1000 cells in at least 5 HPFs and the percentage was estimated. Cases were considered as negative if < 20% and positive if ≥ 20%. | Modified from (Walker and Levine 1996) |
| MIB-1 | The number of positive cells was counted in 1000 cells in at least 5 HPFs and the percentage was estimated. Cases were considered as having low proliferation index if < 20% and having low proliferation index if ≥ 20%. | (Faneyte, Schrama et al. 2003) |
| Bcl-6 | The number of positive cells was counted in 1000 cells in at least 5 HPFs and the percentage was estimated. Cases were considered as negative if < 10% and positive if ≥ 10%. | (Zhang, Ohshima et al. 1999) |
| Bcl-2 | The number of positive cells was counted in 1000 cells in at least 5 HPFs and the percentage was estimated. Cases were considered as negative if < 10% and positive if ≥ 10%. | (Zhang, Ohshima et al. 1999) |

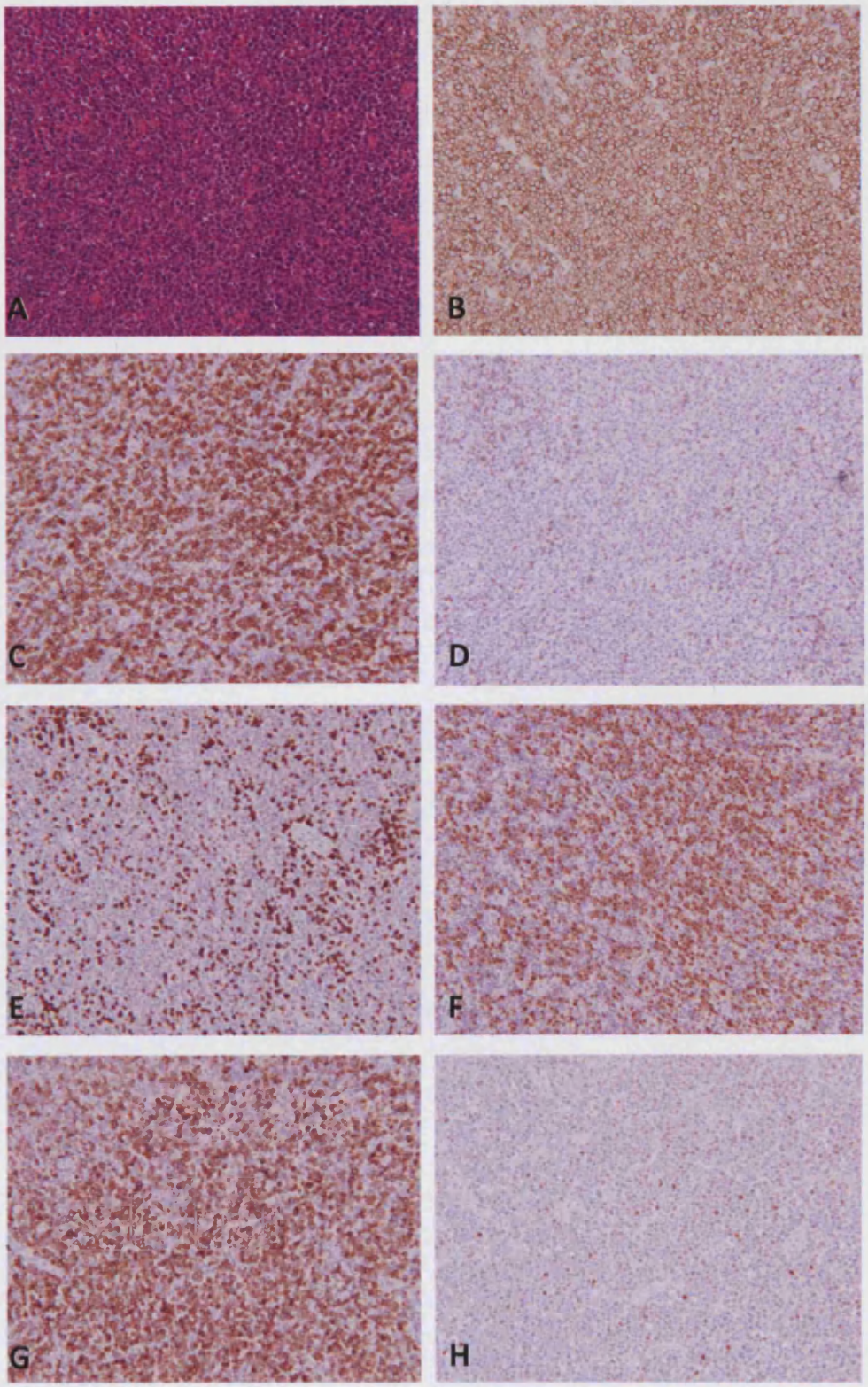



**Figure 3-1: Example of a case of DLBCL.** (A): H&E staining. (B): CD20. (C): CD79a. (D): CD5 (E): CD3. (F): Bcl-6 positive staining. (G): Bcl-2 positive staining. (H): MIB-1 negative.

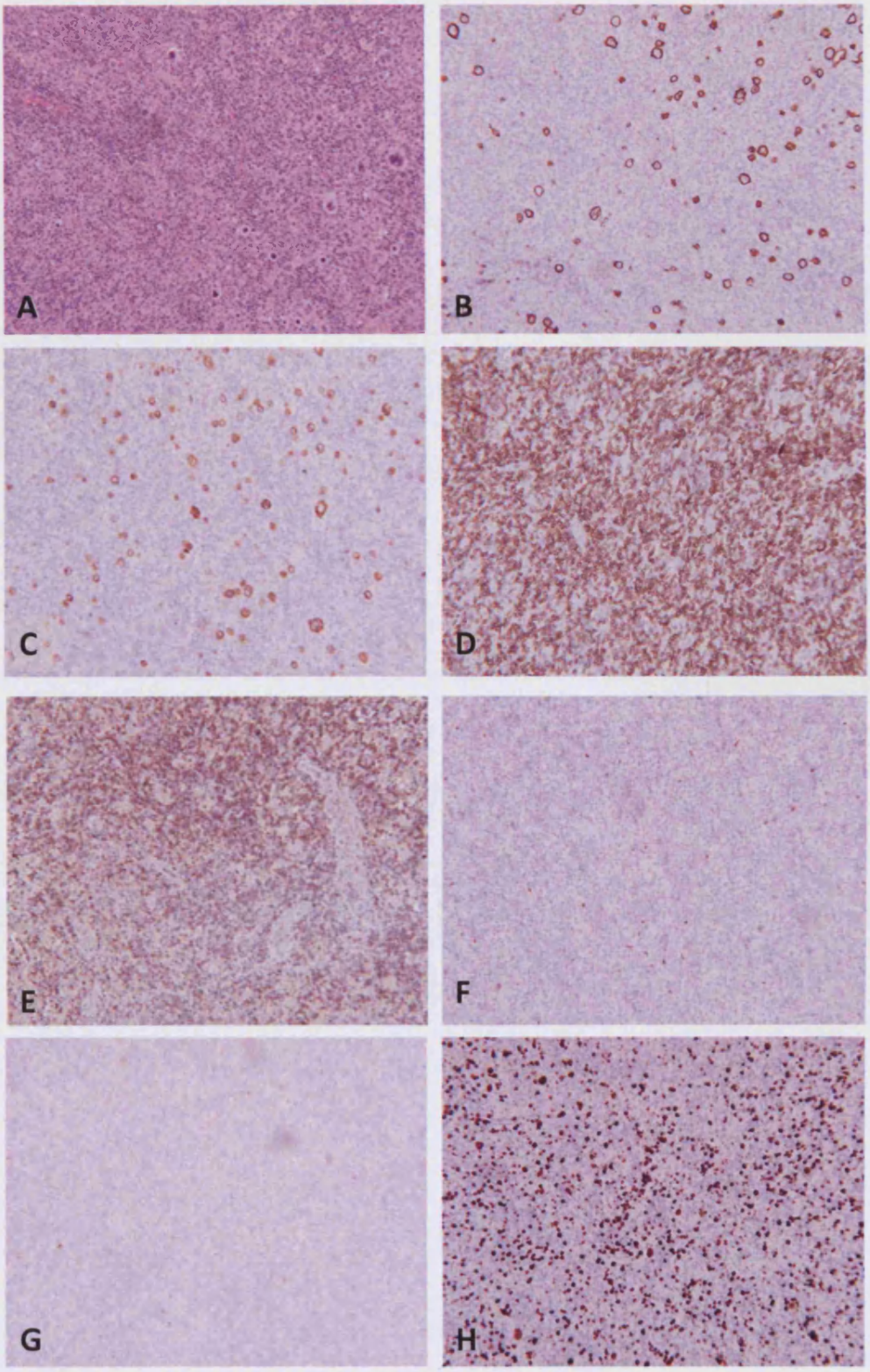
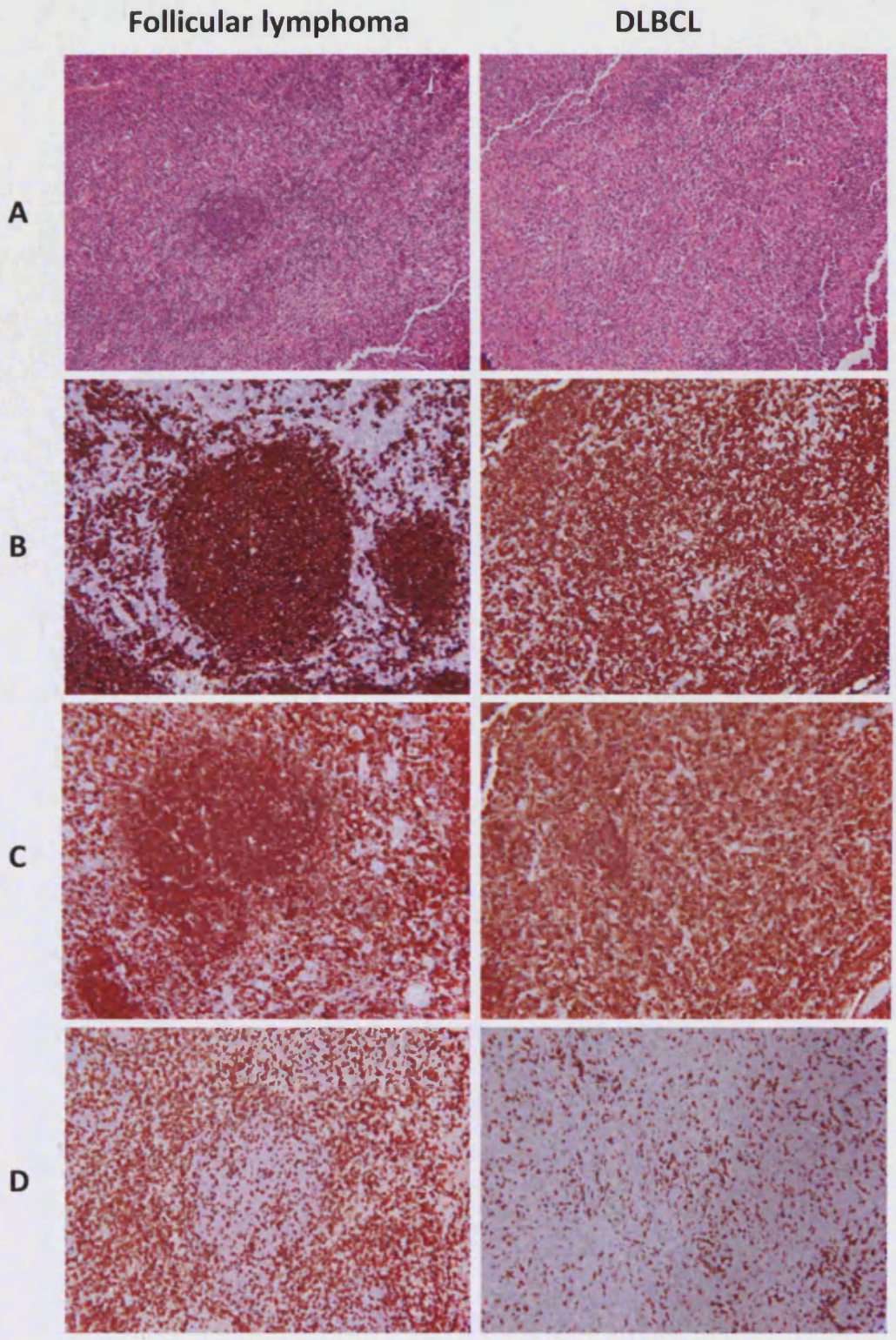



**Figure 3-2: Example of a case belonging to TCR subgroup.** (A): H&E staining. (B): CD20. (C): CD79a. (D): CD5 (E): CD3. (F): Bcl-6 negative staining. (G): Bcl-2 negative staining. (H): MIB-1 positive.


Follicular lymphoma
DLBCL
A
B
C
D

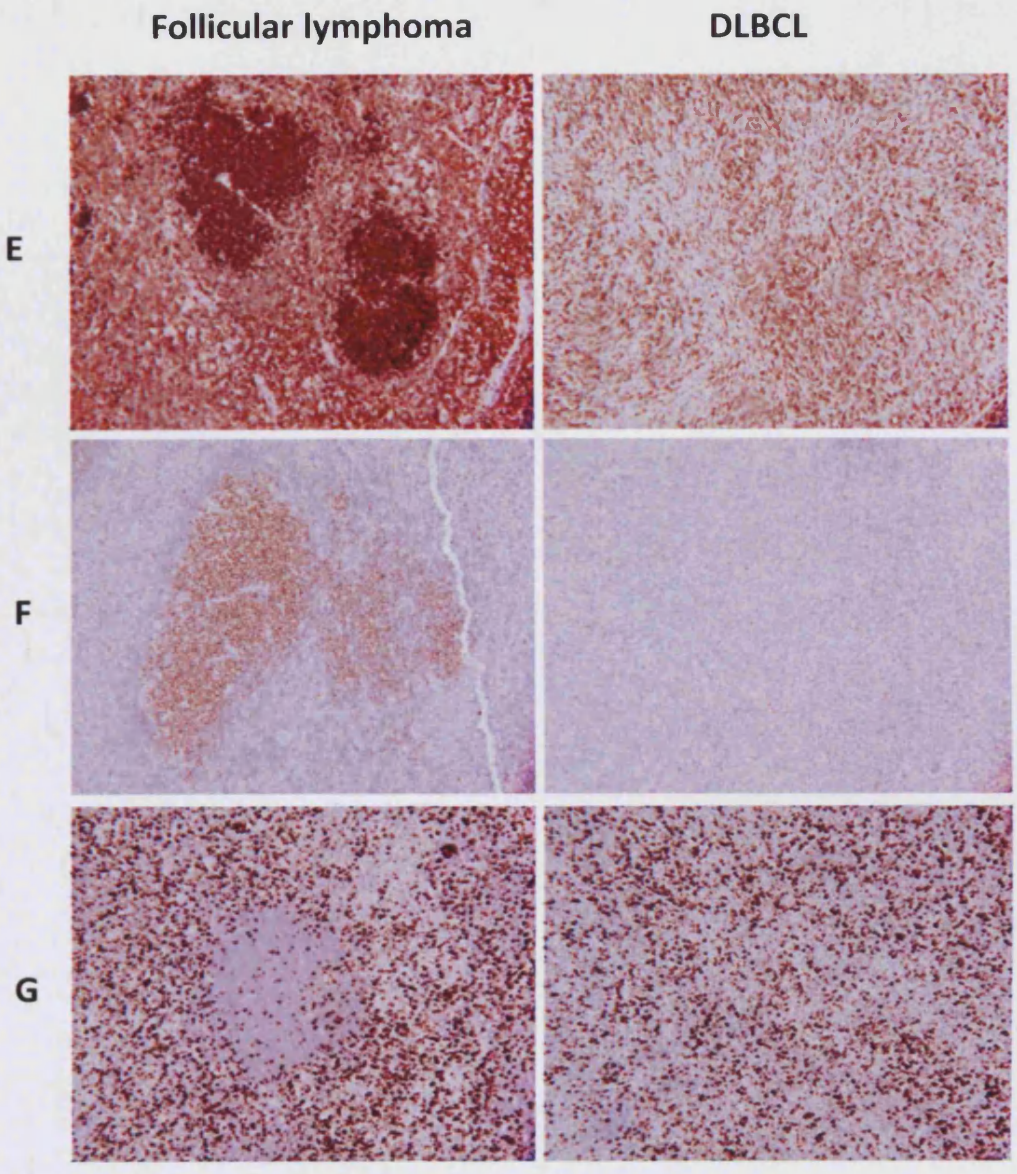



**Figure 3-3: A case of transformed FL into DLBCL**. (A): H&E. (B): CD20. (C): CD79a. (D): CD5. (E): Bcl-2. (F): Bcl-6 .(G): MIB-1.

**Table 3-3: The relationship of the pathologic subtype to other clinico-pathological parameters.**

| *Clinico-pathologic parameter* | | *Diagnosis* | |
|---|---|---|---|
| | | DLBCL | TCR |
| Age | <60 | 24 | 5 |
| | ≥60 | 41 | 2 |
| Gender | M | 34 | 5 |
| | F | 31 | 2 |
| TP53 | - | 33 | 1 |
| | + | 32 | 6 |
| p21 | - | 53 | 1 |
| | + | 12 | 6 |
| HDM2 | - | 53 | 2 |
| | + | 12 | 5 |
| Phospho-p53 | - | 43 | 5 |
| | + | 22 | 2 |
| Bcl-6 | - | 30 | 2 |
| | + | 35 | 5 |
| Bcl-2 | - | 30 | 6 |
| | + | 35 | 1 |
| Total | | 65 | 7 |

## 3.4.4 Immunophenotypic characterisation of DLBCL:

### *3.4.4.1 Proliferation Index:*

Ki 67 antigen (MIB-1) was used as marker of cellular proliferation. The staining was predominantly nuclear and occasionally cytoplasmic. Only nuclear staining in large malignant B-cells was considered for scoring. A cut off of 20 % was used for defining positive immunostaining. MIB-1 staining ranged from 20-95% with a median and SD of 60% and 20.6% respectively. The full data for each case is shown in Table 3-12. Figure 3-1 shows examples of high and low proliferating tumours. The percentage of MIB-1 was calculated in relation to the total B-cells in similar fields.

Proliferation index showed a significant correlation with age (p=0.026), as the mean rank was higher in patients aged 60 years or older. Nevertheless, this correlation was not seen with gender or with the pathological subtype. Proliferation also showed a significant positive correlation with TP53, phospho-TP53 and Bcl-6 (p=0.001, 0.001 and 0.003 respectively), while the mean rank for MIB-1 positivity was significantly higher in Bcl-2 positive cases (p=0.001). Proliferation index showed no correlation with p21 and HDM2 expressions (p=0.7 and 0.337 respectively).

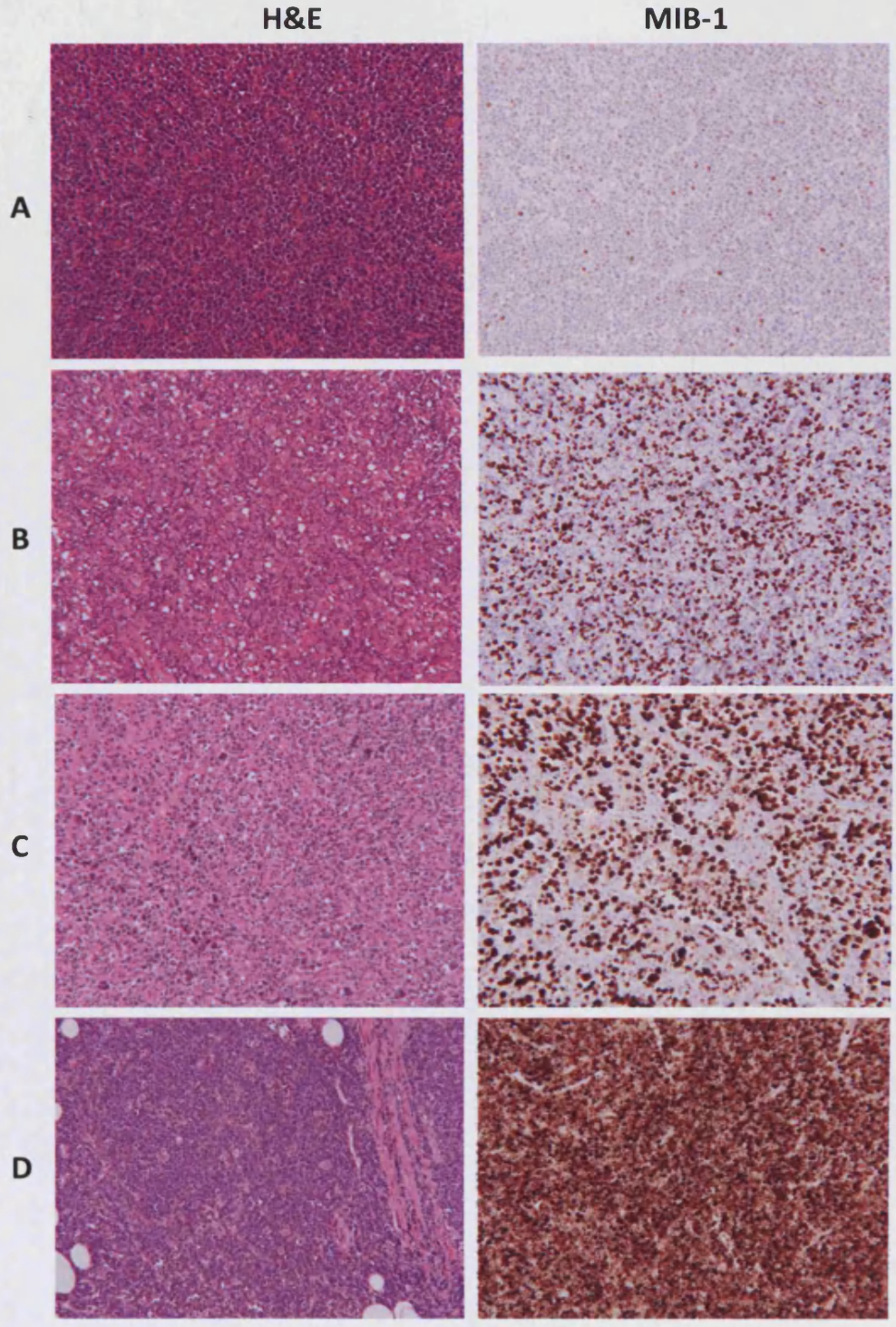



**Figure 3-4: Photomicrographs showing examples of assessment of proliferation index by Ki-67 antibody.** (A): MIB-1 negative staining (<20% of 200 total cells/HPF). (B): MIB-1 positive staining in TCR case (80 % of 80 total cells/HPF). (C): MIB-1 positive staining in DLBCL cases (80% of 200 total cells/HPF). (D): MIB-1 positive staining in DLBCL cases (90% of 200 total cells/HPF).

**Table 3-4: Relationship between proliferation and Clinico-pathologic parameter.**

| *Clinico-pathologic parameter* | | *MIB-1* No | Mean Rank | *p* |
|---|---|---|---|---|
| *Age* | <60 | 29 | 29.90 | 0.026* |
| | ≥60 | 43 | 40.95 | |
| *Gender* | M | 39 | 36.73 | 0.9 |
| | F | 33 | 36.23 | |
| *Diagnosis* | DLBCL | 65 | 36.38 | ND |
| | TCR | 7 | 37.57 | |
| *TP53* | - | 34 | 25.6 | 0.001** |
| | + | 38 | 46.3 | |
| *p21* | - | 54 | 35.98 | 0.7 |
| | + | 18 | 38.06 | |
| *HDM2* | - | 55 | 35.2 | 0.337 |
| | + | 17 | 41.71 | |
| *Phospho-p53* | - | 48 | 28.78 | 0.001** |
| | + | 24 | 51.94 | |
| *Bcl-6* | - | 32 | 28.44 | 0.003** |
| | + | 40 | 42.95 | |
| *Bcl-2* | - | 36 | 54.65 | 0.001** |
| | + | 36 | 27.35 | |

**refers to statistical significance at the 0.05 level, ** refers to statistical significance at the 0.005 level, ND: Not Determined, excluded from statistical testing due to few number of cases.*

### *3.4.4.2 Bcl-6:*

The Bcl-6 antibody staining was confined to B-cells with no staining of plasma cells or macrophages. Therefore, dense micro-granular or diffuse staining only confined to the nucleus was counted. Positive Bcl-6 immunostaining was found in 40/72 of cases (55%). A cut-off of 10% was used to define positive Bcl-6 in DLBCL. Figure 3-4 gives examples of Bcl-6 positive and negative tumours and the corresponding H&E staining.

Bcl-6 expression did not correlate with age, gender, or the pathologic subtype of patients. There was a significant correlation between Bcl-6 and Bcl-2 protein expressions (p=0.001), 29 of 40 tumours with positive Bcl-6 had down-regulated Bcl-2, compared to 7 of 32 with negative Bcl-6. However, no such correlation could be found with TP53 or other immunohistochemical markers. The summary of the findings for Bcl-6 in relation to other markers is summarised in Table 3-5. The full data for each case is shown in Table 3-12.

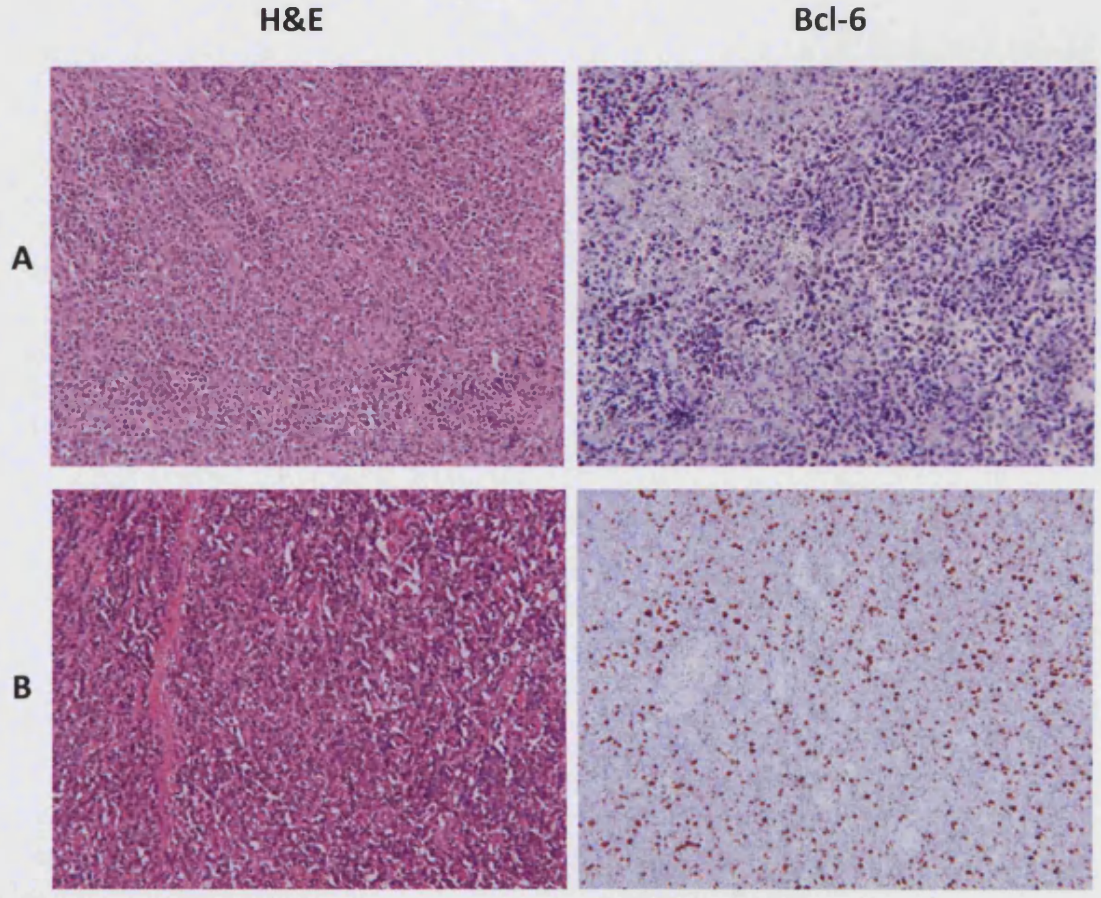



**Figure 3-5: Examples of positive and negative Bcl-6 immunostaining.** (A) Bcl-6 negative immunostaining (<10%). (B) Bcl-6 positive immunostaining (≥ 10%).

**Table 3-5: Bcl-6 and clinico-pathologic parameters.**

| *Clinico-pathologic parameter* | | *Bcl-6* - | *Bcl-6* + | *p* |
|---|---|---|---|---|
| Age | <60 | 13 | 16 | 0.574 |
| | ≥60 | 19 | 24 | |
| Gender | M | 15 | 24 | 0.191 |
| | F | | | |
| Diagnosis | DLBCL | 30 | 35 | ND |
| | TCR | 2 | 5 | |
| TP53 | - | 17 | 17 | 0.255 |
| | + | 15 | 23 | |
| p21 | - | 23 | 31 | 0.391 |
| | + | 9 | 9 | |
| HDM2 | - | 25 | 30 | 0.490 |
| | + | 7 | 10 | |
| Phospho-p53 | - | 23 | 25 | 0.280 |
| | + | 9 | 15 | |
| Bcl-2 | - | 7 | 29 | 0.001** |
| | + | 25 | 11 | |
| Total | | 32 | 40 | |

*** refers to statistical significance at the 0.005 level, ND: Not Determined, excluded from statistical testing due to few number of cases.*

#### *3.4.4.3 Bcl-2:*

The pro-survival protein Bcl-2 showed as cytoplasmic staining. Bcl-2 was positive in 36 of 72 cases (50%). Examples of the different staining patterns for Bcl-2 are given in Figure 3-6. The cytoplasmic Bcl-2 staining was assessed in the whole tumour area in the same fields showing mostly large malignant B-cells and assessed in only malignant B-cells previously highlighted in similar fields by CD20 and CD79a.

There was no correlation between Bcl-2 expression and age or gender. Although there was a tendency for low expression of Bcl-2 in TCR lymphoma subgroup, this did not reach statistical significance ($p=0.053$). There was a significant inverse correlation between Bcl2 and TP53, Phosho-p53 and Bcl-6 ($p<0.005$), while a similar correlation, but to a lesser extent, with HDM2 ($p<0.05$) was observed (Table 3-6). The full data for each case is shown in Table 3-12.

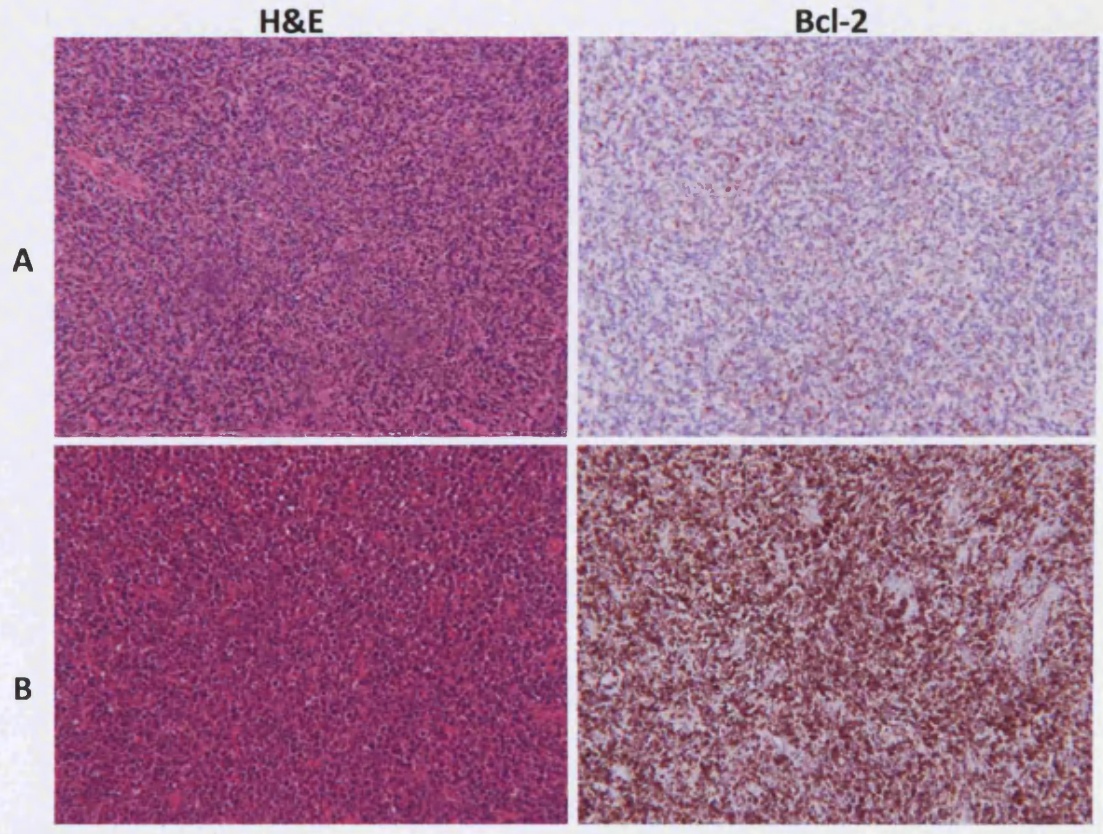



**Figure 3-6: Photomicrographs showing examples of positive and negative Bcl-2 immunostaining** (A): Bcl-2 negative immunostaining (<10%). (B): Bcl-2 positive immunostaining (≥ 10%).

**Table 3-6: Bcl-2 and clinico-pathologic parameters.**

| *Clinico-pathologic parameters* | | *Bcl-2* - | *Bcl-2* + | *p* |
|---|---|---|---|---|
| Age | <60 | 14 | 15 | 0.5 |
| | ≥60 | 22 | 21 | |
| Gender | M | 22 | 17 | 0.172 |
| | F | 14 | 19 | |
| Diagnosis | DLBCL | 30 | 35 | ND |
| | TCR | 6 | 1 | |
| TP53 | - | 11 | 23 | 0.004** |
| | + | 25 | 13 | |
| p21 | - | 24 | 30 | 0.086 |
| | + | 12 | 6 | |
| HDM2 | - | 24 | 31 | 0.047* |
| | + | 12 | 5 | |
| Phospho-p53 | - | 18 | 30 | 0.003** |
| | + | 18 | 6 | |
| Bcl-6 | - | 7 | 25 | 0.001** |
| | + | 29 | 11 | |
| Total | | 36 | 36 | |

**refers to statistical significance at the 0.05 level, ** refers to statistical significance at the 0.005 level, ND: Not Determined, excluded from statistical testing due to few number of cases.*

### 3.4.5 TP53:

Sections were stained with Do-1 monoclonal antibody that recognizes both mutant and WT-p53. Colorectal carcinomas served as a control for this analysis and a cut-off of ≥20% was applied to define immune-positivity. Ten different fields were examined and a percentage was estimated based on the total cells showing B-cell markers; CD20 and CD79 in similar fields. The percentage of staining ranged from 2.2 - 66.7% with a median and SD of 20% 14.75 respectively as shown in Figure 3-6. Thirty four cases (47.2%) showed staining ≤ 20% and were considered negative. Isolated, strong nuclear TP53 staining was found in few cases, this was also considered negative. TP53 nuclear staining ≥20 % was found in 38 cases (52.8%). Examples of TP53 immunostaining are given in Figure 3-7. The full data for each case is shown in Table 3-12.

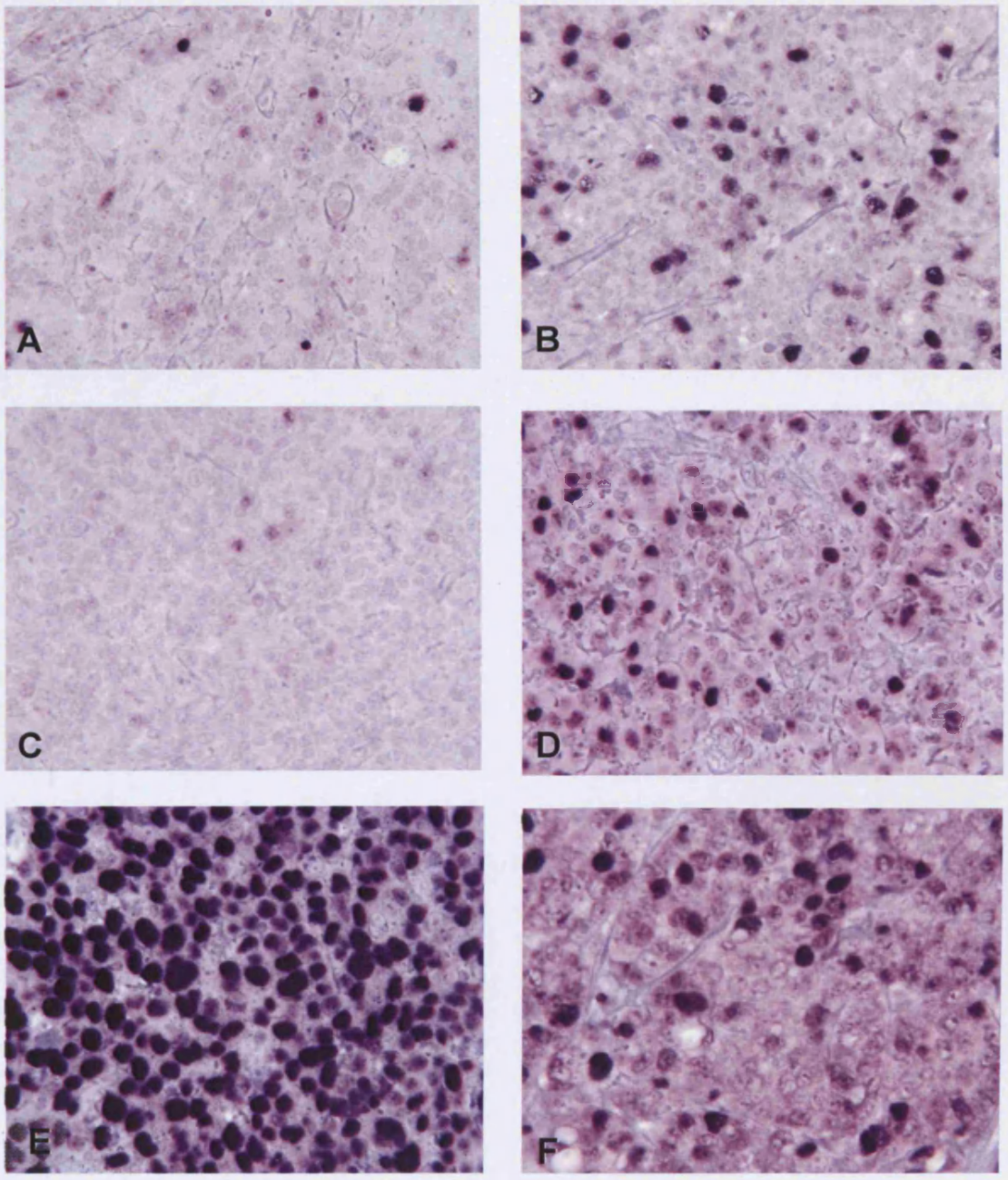



**Figure 3-7: Immunohistochemical staining of TP53 in DLBCL using the DO1 antibody.** Photomicrographs of cases showing nuclear staining with NBT and BCIP substrate: (A): TP53 negative staining in a TCR case (< 20% of 80 cells/HPF) (B): TP53 positive in a TCR case (>20% of 80 cells/HPF). (C): TP53 negative in a DLBCL case (<10% of 200 cells/HPF). (D): TP53 negative in a DLBCL case (<20% of 200 cells/HPF). (E): TP53positive case (> 20%, of 200 cells/HPF). (F): Positive TP53 in colorectal carcinoma.

**TP53 and other biological and immunohistochemical markers:**

Although TP53 protein expression was higher in patients aged 60 years or older (26 positive compared to 17 negative cases for TP53 in older patients), this did not reach statistical significance (p=0.088). There was no correlation between TP53 expression neither with gender nor with pathologic subtype.

Significant correlation was found between TP53 and phospho-p53 staining (r=0.52, p<0.001) as shown in Figure 3-8. All cases stained positive for phospho-p53 also stained positive for TP53 by DO-1. While 34 cases with positive TP53 staining were negative for phospho-p53. On the other hand a strong negative correlation was found between TP53 and Bcl-2 protein expression (p<0.005) as given in Table 3-7.

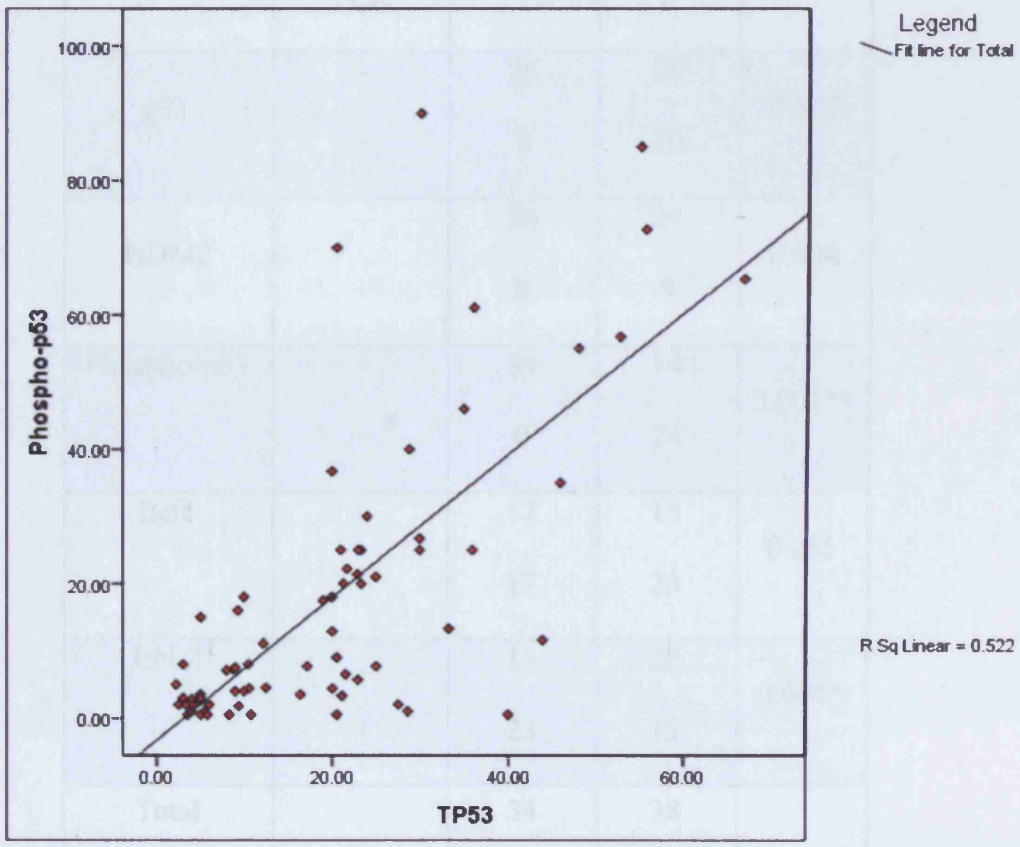



**Figure 3-8: Correlation between TP53 and phospho-specific TP53 (r=0.522, p<0.001).**

**Table 3-7: TP53 and other clinico-pathologic parameters.**

| *Clinico-pathologic parameter* | | *TP53* - | *TP53* + | *p* |
|---|---|---|---|---|
| Age | <60 | 17 | 12 | 0.088 |
| | ≥60 | 17 | 26 | |
| Gender | M | 17 | 22 | 0.332 |
| | F | 17 | 16 | |
| Diagnosis | DLBCL | 33 | 32 | ND |
| | TCR | 1 | 6 | |
| p21 | - | 26 | 28 | 0.501 |
| | + | 8 | 10 | |
| HDM2 | - | 26 | 29 | 0.604 |
| | + | 8 | 9 | |
| Phospho-p53 | - | 34 | 14 | 0.001** |
| | + | 0 | 24 | |
| Bcl6 | - | 17 | 15 | 0.255 |
| | + | 17 | 23 | |
| Bcl-2 | - | 11 | 25 | 0.004* |
| | + | 23 | 13 | |
| Total | | 34 | 38 | |

**refers to statistical significance at the 0.05 level, ** at the level 0.005, ND: Not Determined, excluded from statistical testing due to few number of cases.*

### 3.4.6 p21/WAF1:

p21/Waf1, clone SX118, monoclonal antibody was used to stain sections retrieved from 72 DLBCL cases. Colorectal carcinomas were used as positive control for this antibody and a cut-off of ≥5% was applied to define immune-positivity. Staining for p21 was predominantly nuclear with occasional cytoplasmic staining, only nuclear staining in neoplastic B-cells was considered positive. Expression of p21 was relatively frequent in DLBCL, positive staining (≥ 5%) was found in 24 patients (33.3%). The percentage of nuclei stained positive with p21 antibody ranged from 0.5- 40%, with a mean and median of 5.1 and 3.3 respectively. Examples of p21 immunohistochemical staining are given in Figure 3-9. The full data for each case is shown in Table 3-12.

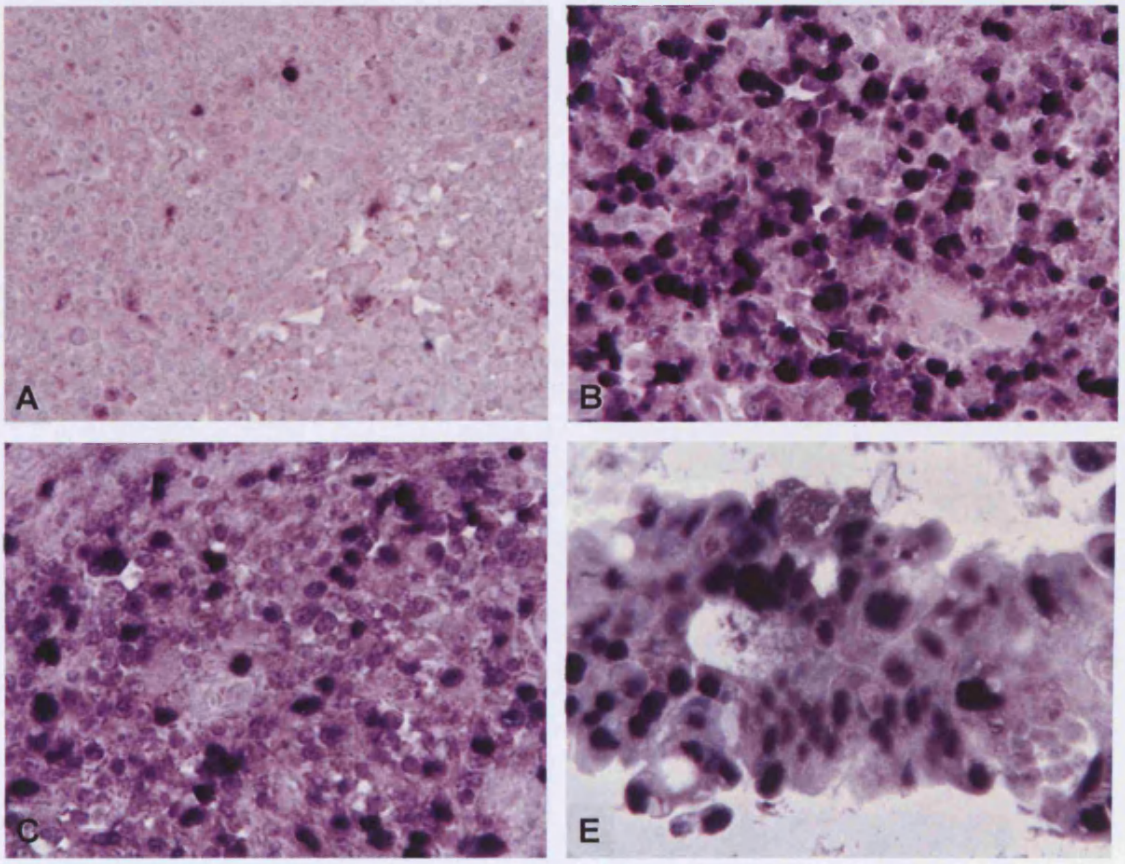



**Figure 3-9: p21 immunohistochemical staining**. Photomicrographs of cases showing nuclear staining with NBT and BCIP substrate. (A): Negative immunostaining in a DLBCL case (<5% of 250 cells/HPF). (B): Positive immunostaining (>5% of 200 cells/HPF). (C): Positive immunostaining in TCR case (>5% of 100 cells/HPF). (D): Positive immunostaining in gastric carcinoma.

**p21 and other biological and immunohistochemical markers:**

Seven cases included in this study belonged to T-cell rich subgroup of lymphomas, p21 staining was found in 6/7 cases (85.7%) of these cases. In cases of DLBCL, p21 was positive in 12 out of 65 cases (18.5%, p=0.001). No significant correlation was found between p21 expression and age or gender. There was a significant correlation between p21 and HDM2 protein expressions (p=0.001), while there was no such correlation with other markers. Although 12 of 18 cases with p21 positivity had low expression of Bcl-2, this did not reach statistical significance (p=0.086), as shown in Table 3-7.

**Table 3-8: p21/Waf1 and other biological and clinico-pathologic parameter.**

| Clinico-pathologic Parameter | | p21 - | p21 + | p |
|---|---|---|---|---|
| Age | <60 | 22 | 7 | 0.559 |
| | ≥60 | 32 | 11 | |
| Gender | M | 30 | 9 | 0.444 |
| | F | 24 | 9 | |
| Diagnosis | DLBCL | 53 | 12 | ND |
| | TCR | 1 | 6 | |
| TP53 | - | 26 | 8 | 0.501 |
| | + | 28 | 10 | |
| HDM2 | - | 47 | 8 | 0.001** |
| | + | 7 | 10 | |
| Phospho-p53 | - | 37 | 11 | 0.381 |
| | + | 17 | 7 | |
| Bcl6 | - | 23 | 9 | 0.391 |
| | + | 31 | 9 | |
| Bcl-2 | - | 24 | 12 | 0.086 |
| | + | 30 | 6 | |
| Total | | 54 | 18 | |

**refers to statistical significance at the 0.05 level, ** at the level 0.005, ND: Not Determined, excluded from statistical testing due to few number of cases.*

### 3.4.7 Establishment of TP53/p21 phenotype:

p21 is a WT-p53 inducible gene, located at chromosome 6p. Mutations of *TP53* result in the production of a protein with an abnormal structure and prolonged half-life that is detectable by immunohistochemical methods. This mutant TP53 is unable to induce p21-WAF1 expression and hence the presence of TP53+/p21- would point out non functional TP53.

To describe the complex distribution of p21 and its relationship with TP53 expression, four patterns of staining were defined. TP53+/p21- was the commonest pattern found in 28 cases (38.9%) followed by a double negative pattern; TP53-/p21- in 26 cases (36.1%). Double positive pattern; TP53+/p21+, was found in 10 patients (13.9%). Neoplastic cells expressed p21 with lack of TP53 expression; TP53-/p21+, in eight patients (11.1%), this represented the least common phenotype as shown in Table 3-9 and Figure 3-8. Examples of different TP53/p21 phenotypes assessed by immunohistochemistry are shown in Figure 3-9.

**Table 3-9: Frequency of the four TP53/p21 patterns.**

| | | TP53 | |
|---|---|---|---|
| p21 | | Positive | Negative |
| | Positive | 10 (13.9%) | 8 (11.1%) |
| | Negative | 28 (38.9%) | 26 (36.1%) |


**Figure 3-8: Venn diagram depicting the different TP53/p21 expression patterns.**

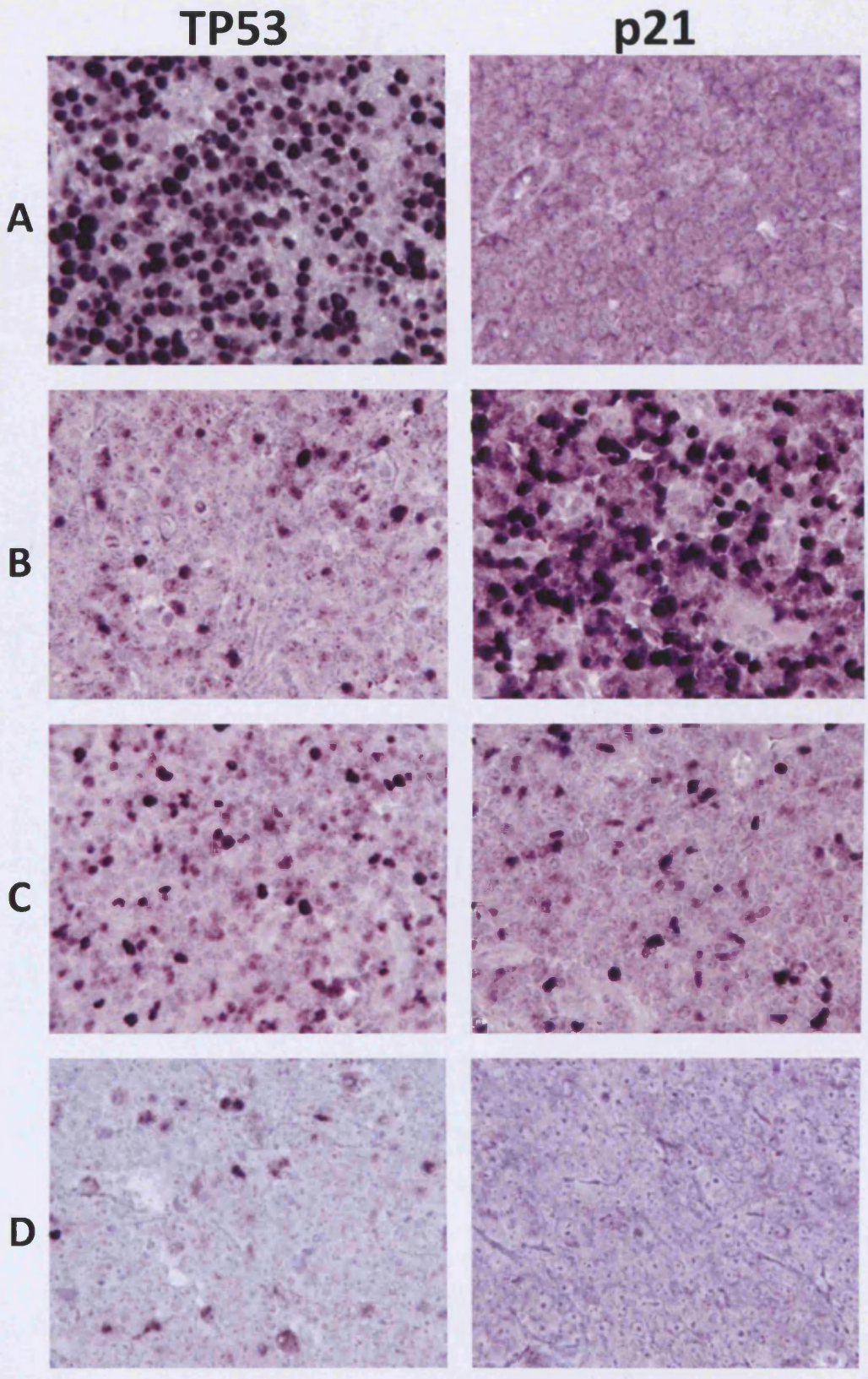



**Figure 3-9: TP53/p21 expression patterns in DLBCL.** (A): TP53+/p21-, (B): TP53-/p21+ in DLBCL, (C): TP53+/p21+ in TCR lymphoma, (D): TP53-/p21- in DLBCL.

### 3.4.8 Phospho-specific TP53:

NCL-p53-CMI monoclonal antibody was used to assess phospho-p53 which is phosphorylated at Serine 392. Colorectal carcinomas were used as positive control for this antibody. Positive staining for phospho-p53 (≥ 20%) was detected in 24 cases (33.3%). The percentage of phospho-TP53 ranged from 0.5- 90% with a median and SD of 7.8 and 21.6366 respectively. Examples of Phospho-TP53 imuunohistochemical staining is shown in Figure 3-10. The full data for each case is shown in Table 3-12.

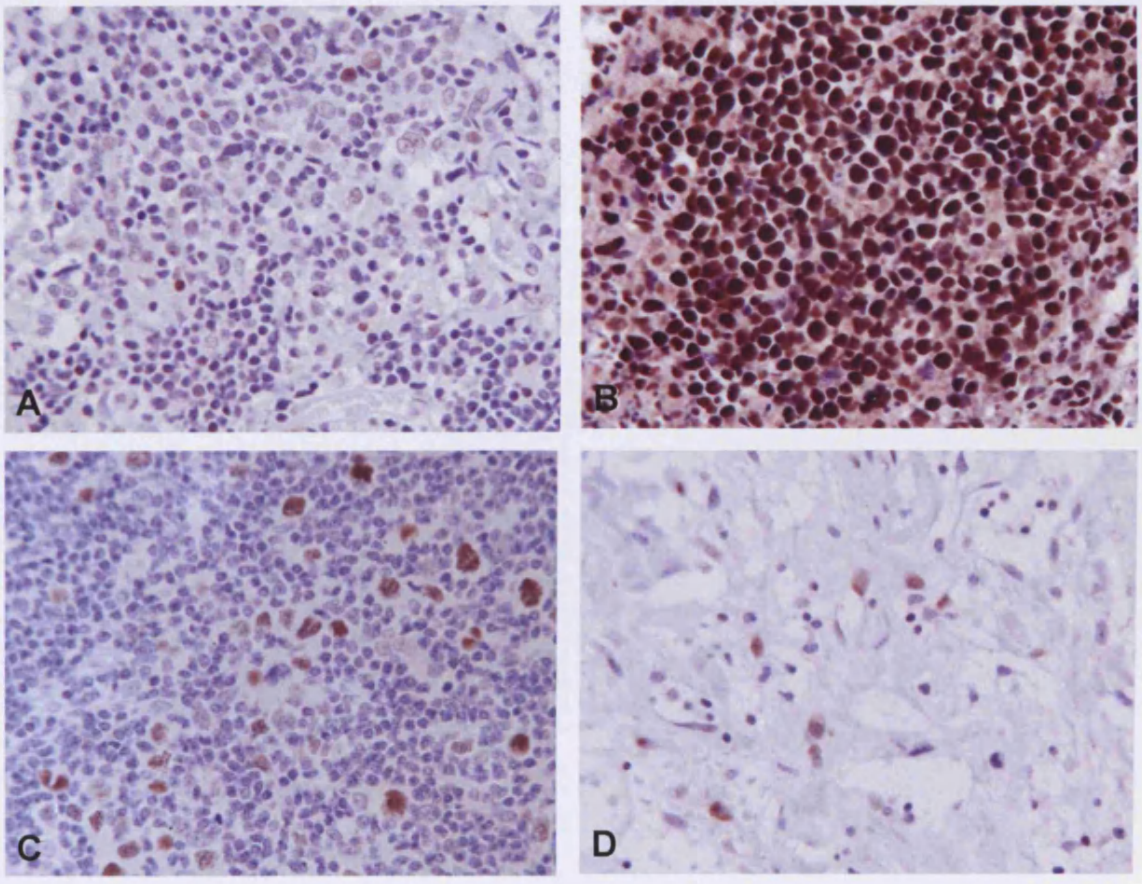



**Figure 3-10: Phospho-p53 immunohistochemical staining.** (A): Negative immunostaining (> 20% of 70 cells/HPF). (B): Positive immunostaining in classic DLBCL case (> 20% of 250 cells/HPF). (C): Positive immunostaining in TCR case (> 20% of 70 cells/HPF). (D): Positive immunostaining in colorectal carcinoma.

**Phospho-p53 and other biological and immunohistochemical markers:**

A significant correlation was found between phospho-p53 and older age of the patients (60 years or older, $p<0.05$). However, there was no correlation between phospho-p53 and gender or with the pathological subtype. No correlation was found between p21 and phospho-p53 ($p=0.57$) and with HDM2 ($p=0.468$). A significant negative correlation was found between phospho-p53 and Bcl-2 ($p=0.005$), 18 of 24 tumours with positive phopho-p53 had down-regulated Bcl-2. There was no correlation between Bcl-6 and TP53 phosphorylated at serine 392 ($p=0.28$), which was similar to the finding noted to TP53 detected by DO-1 ($p=0.26$), as shown in Table 3-10.

**Table 3-10: Phospho-p53 and other biological and immunohistochemical markers.**

| *Clinico-pathologic parameter* | | *Phospho-p53* - | + | *p* |
|---|---|---|---|---|
| Age | <60 | 24 | 5 | 0.022* |
| | ≥60 | 24 | 19 | |
| Gender | M | 25 | 4 | 0.402 |
| | F | 32 | 10 | |
| Diagnosis | DLBCL | 43 | 22 | ND |
| | TCR | 5 | 2 | |
| TP53 | - | 34 | 0 | 0.001** |
| | + | 14 | 24 | |
| p21 | - | 37 | 17 | 0.576 |
| | + | 11 | 7 | |
| HDM2 | - | 36 | 19 | 0.468 |
| | + | 12 | 5 | |
| Bcl6 | - | 23 | 9 | 0.28 |
| | + | 25 | 15 | |
| Bcl-2 | - | 18 | 18 | 0.005** |
| | + | 30 | 6 | |
| Total | | 48 | 24 | |

**refers to statistical significance at the 0.05 level, ** at the level 0.005, ND: Not Determined, excluded from statistical testing due to few number of cases.*

### 3.4.9 HDM2:

HDM2 protein expression was assessed in 72 DLBCL using SMP14 on paraffin embedded sections. A cut-off of ≥ 10% was applied. Although some occasional cytoplasmic staining was found in some cases, only nuclear staining was considered positive. Breast carcinomas were used as positive controls in this study. Positivity of neoplastic B-cells for HDM2 was found in 23.6% and ranged from 0.5-33.3%, with a median and SD of 4 and 8.3 respectively. Examples of different staining patterns are shown in Figure 3-12. The full data for each case is shown in Table 3-11.

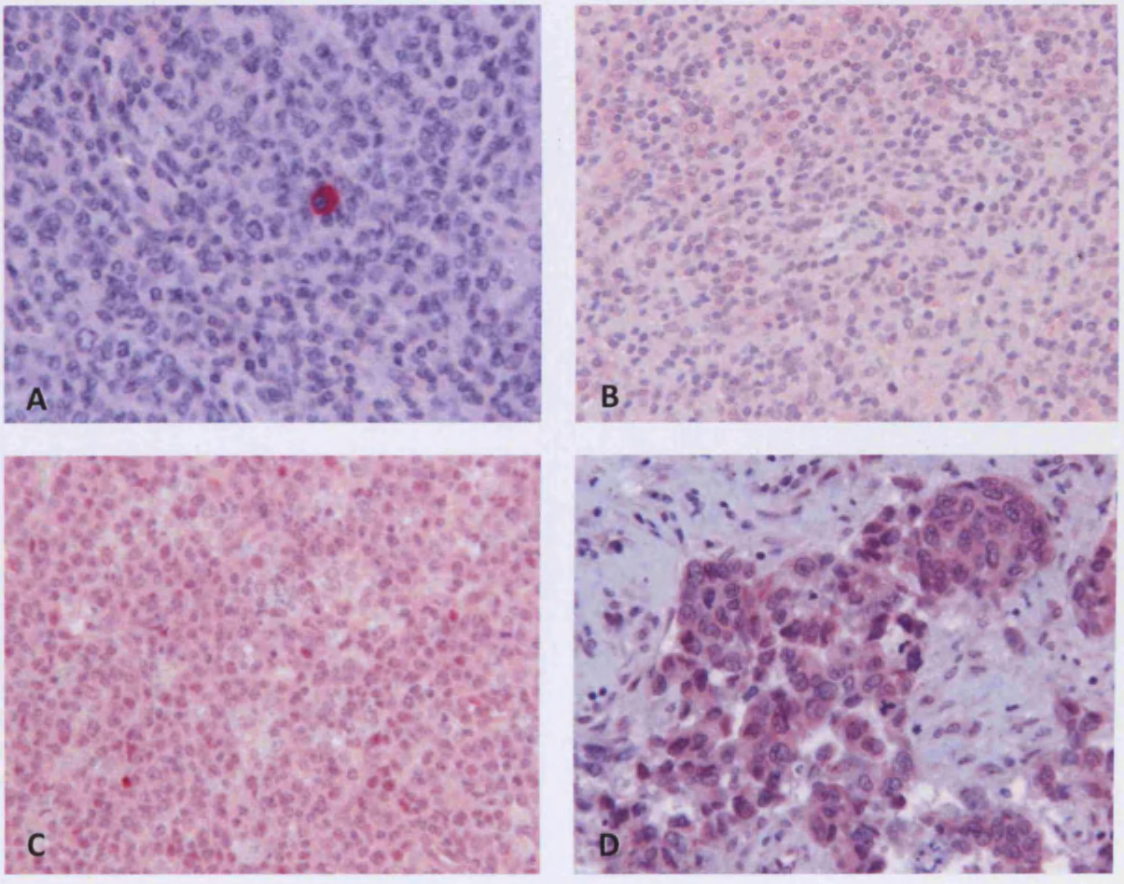



**Figure 3-12: Photomicrograph of HDM2 immunohistochemical staining.** (A): Negative immunostaining in DLBCL. (B): Positive immunostaining in DLBCL. (C): Positive immunostaining in TCR case. (D): Positive immunostaining in breast carcinoma.

**HDM2 and other biological and immunohistochemical markers:**

Like p21, HDM2 expression was higher in T-cell rich subgroup. Among 7 cases of TCR, HDM2 was positive in 5 cases (71.4%) compared to 12/65 cases (18.46%) of DLBCL. But there was no correlation between age and gender (p=0.175 and 0.437 respectively). There was a significant correlation between HDM2 and p21 expression (r=0.3337, p<0.005). A significant correlation was also found between HDM2 expression and Bcl-2 negativity (p<0.05). While no correlation was found between HDM2 and TP53 or phospo-TP53 (p=0.9 and 0.7 respectively) as shown in Table 3-12.

**Table 3-11: HDM2 and other biological and immunohistochemical markers.**

| *Clinico-pathologic parameter* | | *HDM2* - | + | *p* |
|---|---|---|---|---|
| Age | <60 | 20 | 9 | 0.175 |
| | ≥60 | 30 | 8 | |
| Gender | M | 29 | 10 | 0.437 |
| | F | 26 | 7 | |
| Diagnosis | DLBCL | 53 | 12 | ND |
| | TCR | 2 | 5 | |
| TP53 | - | 26 | 8 | 0.604 |
| | + | 29 | 9 | |
| p21 | - | 47 | 7 | 0.001** |
| | + | 8 | 10 | |
| Phospho-p53 | - | 36 | 12 | 0.468 |
| | + | 19 | 5 | |
| Bcl6 | - | 25 | 7 | 0.49 |
| | + | 30 | 10 | |
| Bcl-2 | - | 24 | 12 | 0.047* |
| | + | 31 | 5 | |
| Total | | 55 | 17 | |

**refers to statistical significance at the 0.05 level, ** at the level 0.005, ND: Not Determined, excluded from statistical testing due to few number of cases.*

### 3.4.10 Summary of the results:

Seventy-two cases of DLBCL were assessed by immunohistochemistry using primary antibodies for TP53, p21, HDM2, Phospho-TP53, Bcl-2, Bcl-6 and MIB-1. Seven cases of them belonged to the TCR lymphoma subgroup. There was a significant correlation between the older age of the patients ($\geq$ 60 years) and MIB-1 and phospho-TP53 over-expression but not with the other markers. The gender of the patients did not show a statistically significant correlation with any of the markers.

There was a significant positive correlation between high proliferation index and TP53, phopho-TP53 and Bcl-6 expressions ($p<0.005$), while a significant negative correlation was found with Bcl-2 expression ($p<0.005$). Bcl-2 expression showed an inverse correlation with TP53, phospho-TP53, Bcl-6 ($p<0.005$ each) and HDM2 ($p<0.05$). TP53 assessed using the DO-1 antibody showed a significant correlation with phopho-TP53 ($p<0.005$), but not with p21 or HDM2. p21 showed a similar correlation with HDM2 ($p<0.005$). The full data for individual cases is given in Table 3-12.

**Table 3-12: Collective data of TP53 and other related protein assessed by IHC.**

| *Cases ID* | *p21* | *TP53* | *Phospho-p53* | *HDM2* | *MIB-1* | *Bcl-2* | *Bcl-6* |
|---|---|---|---|---|---|---|---|
| 1 | 10 | 8.3 | 0.5 | 16 | 50 | + | - |
| 2 | 8.2 | 8.8 | 7.2 | 10 | 99 | + | + |
| 3 | +.2 | 40 | 0.5 | 2.8 | 90 | + | - |
| 4 | 4 | 4 | 2.8 | 4 | 40 | + | - |
| 5 | 2.5 | 3.5 | 0.5 | 6 | 50 | - | + |
| 6 | +.2 | 36 | 25 | 2 | 60 | + | - |
| 7 | 4 | 2.5 | 2 | 1 | 75 | + | + |
| 8 | 8.8 | 5.5 | 1 | 4 | 30 | + | + |
| 9 | 0.8 | 44 | 11.6 | 4 | 80 | + | - |
| 10 | 3.6 | 24 | 30 | 4.8 | 60 | + | - |
| 11 | 1.5 | 5 | 0.5 | 3 | 50 | + | + |
| 13 | 5 | 6 | 2 | 4 | 40 | - | - |
| 14 | 4 | 20 | 18 | 4 | 30 | + | - |
| 15 | 8.3 | 30 | 26.7 | 10 | 70 | + | - |
| 16 | 3.3 | 17.2 | 7.7 | 9 | 25 | + | - |
| 17 | 0.6 | 55.6 | 72.7 | 1.1 | 80 | + | - |
| 18 | 0.6 | 36.1 | 61.1 | 1.1 | 70 | + | - |
| 19 | 3.5 | 5 | 15 | 1 | 50 | - | + |
| 20 | 2.5 | 4 | 2 | 0.5 | 50 | - | + |
| 21 | 1.7 | 5.8 | 0.5 | 0.8 | 25 | + | - |

| *Cases ID* | *p21* | *TP53* | *Phospho-p53* | *HDM2* | *MIB-1* | *Bcl-2* | *Bcl-6* |
|---|---|---|---|---|---|---|---|
| 22 | 20.6 | 9.4 | 1.8 | 0.5 | 50 | + | - |
| 23 | 3.3 | 25 | 7.7 | 0.5 | 80 | + | + |
| 24 | 3.8 | 10.6 | 4.4 | 1.5 | 50 | + | - |
| 25 | 1.5 | 55 | 85 | 0.5 | 70 | + | - |
| 26 | 2.7 | 20.5 | 0.5 | 1.1 | 40 | - | + |
| 27 | 1 | 5 | 2.5 | 0.5 | 50 | + | - |
| 28 | 3 | 2.2 | 5 | 1 | 50 | + | - |
| 29 | 3.5 | 21.5 | 6.5 | 2 | 50 | - | - |
| 30 | 0.5 | 5 | 3.5 | 1 | 80 | + | - |
| 31 | 0.5 | 27.5 | 2 | 1 | 90 | + | + |
| 32 | 4 | 20.5 | 9 | 2 | 80 | - | + |
| 33 | 35.7 | 28.6 | 1 | 11.4 | 50 | - | + |
| 34 | 2.8 | 21.7 | 22.2 | 0.5 | 80 | - | - |
| 35 | 1 | 46 | 35 | 1 | 75 | + | - |
| 36 | 3.3 | 10.8 | 0.5 | 3.3 | 30 | - | - |
| 37 | 14.3 | 22.9 | 21.4 | 2.8 | 80 | - | + |
| 38 | 2 | 66.7 | 65.3 | 0.6 | 75 | - | - |
| 39 | 4.6 | 8 | 7.1 | 33.3 | 50 | - | - |
| 40 | 4 | 3.3 | 2 | 7 | 50 | + | + |
| 41 | 4 | 10.5 | 8 | 7 | 60 | + | + |
| 42 | 3 | 10 | 18 | 19 | 85 | + | - |
| 43 | 1.3 | 9.3 | 16 | 33.3 | 70 | + | + |

| *Cases ID* | *p21* | *TP53* | *Phospho-p53* | *HDM2* | *MIB-1* | *Bcl-2* | *Bcl-6* |
|---|---|---|---|---|---|---|---|
| 44 | 18.3 | 33.3 | 13.3 | 20 | 80 | + | - |
| 45 | 10 | 52.7 | 56.7 | 6.6 | 75 | - | - |
| 46 | 3.5 | 12.5 | 4.5 | 8 | 80 | + | - |
| 47 | 8.6 | 20 | 12.9 | 28 | 40 | + | + |
| 48 | 6.4 | 28.8 | 40 | 3.2 | 50 | - | - |
| 49 | 0.71 | 16.4 | 3.5 | 25 | 75 | + | - |
| 50 | 40 | 19 | 17.5 | 18 | 95 | + | - |
| 51 | 1.5 | 5 | 3 | 4 | 75 | + | - |
| 52 | 2 | 30 | 90 | 3 | 75 | - | + |
| 53 | 3.3 | 21.3 | 20 | 13.3 | 90 | + | - |
| 54 | 0.5 | 25 | 21 | 2.5 | 90 | + | + |
| 55 | 0.5 | 20.5 | 70 | 1 | 80 | + | + |
| 56 | 3.3 | 20 | 4.4 | 5 | 20 | + | + |
| 57 | 1.11 | 21.1 | 3.3 | 30 | 50 | + | + |
| 58 | 1.5 | 30 | 25 | 6 | 70 | + | + |
| 59 | 0.5 | 10 | 4 | 9 | 90 | + | + |
| 60 | 1 | 3 | 3 | 2 | 80 | - | + |
| 61 | 4 | 3 | 8 | 4 | 50 | + | + |
| 62 | 3 | 12.2 | 11.1 | 5.5 | 60 | + | + |
| 63 | 5 | 9 | 4 | 4 | 80 | + | + |
| 64 | 2 | 4 | 1 | 5 | 90 | - | + |
| 65 | 7.3 | 23.3 | 20 | 14.6 | 90 | - | + |

| *Cases ID* | *p21* | *TP53* | *Phospho-p53* | *HDM2* | *MIB-1* | *Bcl-2* | *Bcl-6* |
|---|---|---|---|---|---|---|---|
| 66 | 5 | 20 | 36.7 | 6.6 | 90 | - | + |
| 67 | 0.5 | 21 | 25 | 5 | 80 | + | - |
| 68 | 8.3 | 23.3 | 25 | 10 | 90 | - | - |
| 69 | 4.2 | 22.9 | 5.7 | 8 | 75 | + | + |
| 70 | 1 | 23 | 25 | 30 | 65 | - | - |
| 71 | 8 | 9 | 7.5 | 12.5 | 75 | - | + |
| 72 | 2.5 | 35 | 46 | 6 | 70 | + | - |
| 73 | 0.5 | 48 | 55 | 4 | 90 | + | - |

*For TP53, p21, HDM2, MIB-1 and phospho-p53 the score given represents the percentage of B-cells stained with antibody in relation to the total B-cells in similar fields. Bcl-6 and Bcl-6 were assessed as positive >10% or negative <10%.*

## *3.5 Discussion:*

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoma, comprising 30% of all non-Hodgkin's lymphoma. It has many heterogeneous clinical features and varies markedly in response to treatment. The marked molecular heterogeneity that underlies the aggressive behaviour of these tumours could provide important aspects to the understanding and can improve the prognostic predictability as well (Wright, Tan et al. 2003).

This part of the study involved 72 cases of DLBCL, 39 males and 33 females, aged 17-89 at diagnosis. TP53 over-expression (≥ 20%) was observed in 52.8% of DLBCL cases. The frequency of TP53 expression, when assesed using immunohistochemistry is disparate, ranging from 27% (Leroy, Haioun et al. 2002) to 56% (Paik, Jeon et al. 2005). This can be the result of many factors such the antigen retrieval methods and the cut-offs adopted. Also the effect of storage and the fixative used (Prioleau and Schnitt 1995; Jacobs, Prioleau et al. 1996; Bertheau, Cazals-Hatem et al. 1998; van den Broek and van de Vijver 2000).

There was no correlation between the gender of the patients and any of the immunhistochemical markers. The older age of the patients (≥60 years) showed a correlation with MIB-1 ($p< 0.05$) and phospho-p53 ($p<0.05$), but not with other markers, the lack of correlation between TP53 and the biological factors such as age and gender have been previuosly reported (Lan, Chang et al. 2007; Li, Jiang et al. 2008). The absence of such correlation was also shown regarding TP53 expression as well as polymorphisms of *HDM2* and *TP53* genes (Zainuddin, Berglund et al. 2009).

The proliferative indices of NHLs are useful prognostic indicators and provide information independent of other histological and clinical variables (Hall, Levison et al. 1990). In this series, MIB-1 staining ranged from 20-95%. A strong correlation was found between the proliferation index and TP53, Phospho-p53 and Bcl-6. This in concordance with previous studies showing similar results in regards to TP53 and MIB-1 strong correlation so that TP53 over-expression in highly proliferating tumours could be concluded (Cesarman, Inghirami et al. 1993; Pescarmona, Pignoloni et al. 2001).

Bearing in mind that TP53 over-expression can be detected in begnin and malignant haematopoietic cells that are rapidly proliferating (Danova, Giordano et al. 1990).

There was a negative correlation between the proliferation index and the anti-apoptotic protein Bcl-2 ($p<0.005$). A similar finding was shown by other groups (Bai, Agnantis et al. 2003; Jerkeman, Anderson et al. 2004), keeping the notion that anti-apoptotic can inhibit cellular proliferation (Janumyan, Sansam et al. 2003). While a clear relationship was not observed in a recent study examining paediatric DLBCL (Miles, Raphael et al. 2008).

*BCL6* is a key gene for GC formation, and deregulation of *BCL6* can lead to B-cell lymphoma (Cattoretti, Angelin-Duclos et al. 2005). Bcl-6 is a *TP53* transcriptional target (Margalit, Amram et al. 2006) and at the same time, BCL-6 can suppress TP53 expression in GC. This important function of Bcl-6 as a TP53 repressor is crucial to allow GC B-cells to tolerate the physiological DNA breaks required for immunoglobulin class switch recombination and somatic hyper-mutation (Phan and Dalla-Favera 2004).

Recent evidences have indicated that BCL-6 constitutive activation represses TP53 by binding two specific DNA sites within the *TP53* promoter region. Bcl-6 protein expression was positive in this study no correlation was found between TP53 and BCL-6 expressions assessed by immunohistochemistry ($p=0.25$). Similar results were reported by other groups (Visco et al 2006). This finding could be explained by the presence of *TP53* mutations or other pathways exerted by the TP53 which can override this suppressor effect of Bcl-6. The other possible explanation is that Bcl-6 is regulated by an evolutionarily conserved TP53 response element that is frequently disrupted in NHL (Margalit et al 2006).

The resistance to apoptosis is an important factor in lymphomagenesis (Hermine, Haioun et al. 1996). In this series Bcl-2, the main anti-apoptotic protein, over-expression was found in 50% of the cases. There was an inverse correlation between the expression of Bcl-2 and MIB-1, Bcl-6 and HDM2. A similar inverse correlation was previously reported in some studies (Raible, Hsi et al. 1999), but not in other studies

(Skinnider, Horsman et al. 1999). There was also a strong negative correlation between Bcl-2 and TP53 and phospho-p53 expressions. This finding is similar to other groups who showed that WT-p53 down-regulate the anti-apoptotic Bcl-2 protein and the loss of this repression may lead to up-regulation of Bcl-2 (Miyashita, Harigai et al. 1994). Similar finding was also reported in FL, gastric lymphoma (Nakamura, Akazawa et al. 1996) and primary large cell lymphoma of the bone (Huebner-Chan, Fernandes et al. 2001).

p21 protein product is believed to block cyclin/cyclin-dependent kinase (CDK) complex activity and thus prevents the passage of cycling cells from the G1 to the S phase in a TP53-dependent or TP53-independent manner. Expression of p21 was relatively frequent in DLBCL (33%), which is higher than the frequency of 19% reported by other groups (Chilosi, Doglioni et al. 1996). This could be due to the low cut-off value, adopted in this series, for p21 scoring (5% cut-off compared to 10% used in other studies). Another explanation for this high frequency is the inclusion of TCR subgroup in the analysis. Among classic DLBCL, p21 was positive in 12 of 65 patients (18.5%).

There was a strong tendency for p21 over-expression in cases belonging to TCR subgroup. This could be a bias from the small number of patients belonging to this group or could be a true finding curtailed by the different pathologic profile of TCR lymphomas. In TCR lymphomas, the neoplastic B-cells are scattered among large population of T-cells or histiocytes. Occasional tumour cells have been shown to receive stimulatory signals from surrounding inflammatory cells via cytokines secreted or direct cell to cell signalling. There was no correlation between p21 expression and biological parameters like age and gender of the patients

To further stratify the cases examined according to the functional status of TP53, four different TP53/p21 patterns were analysed. TP53+/p21- was the commonest pattern found in 28 cases (38.9%) , in agreement with other reports showing a frequency of 31% for this pattern.(Visco, Canal et al. 2006). While other groups showed a lower frequency (26.31%) (Villuendas, Pezzella et al. 1997). This could be due the higher frequency of p21 in this study, for the above mentioned reasons. The least frequent

pattern was TP53-/p21+ (11.1%), this could be the result of TP53- independent expression of p21 as seen in other types of cancer (DiGiuseppe, Redston et al. 1995). This was also found in normal cells and tissues and is related to cell differentiation.

Double negative phenotype (TP53-/p21-) was seen in 36.1% of cases, this could be explained by presence of TP53 deletion or null mutation with result in absent or truncated protein incapable of activate p21 transcription. A similar finding but with less frequency was reported by (Villuendas, Piris et al. 1992). The other possibility in these cases is that TP53 is wild type and hence under tight check of HDM2 and other ubquitin ligases or lack of signals activating TP53 itself. Double positive pattern (TP53+/p21+) was found in 13.9% of cases. This can indicate a transcriptionally active TP53.

Post-translational modifications of TP53 represent an important mechanism for the regulation and stabilisation of TP53. Phosphorylation-related function has been demonstrarted to affect sequence-specific DNA binding (Wang and Prives 1995) and transcriptional activities of TP53 (Hall, Campbell et al. 1996). In this study, all phospho-p53 positive tumours were positive for TP53 protein expression detected by DO-1. Yap et al 2004 have shown that phosphorylation of TP53 at serine 392 phosphorylation had a clear effect on the anti-apoptotic function of mutant TP53, which was independent of the DNA binding activity of TP53.

In this study, phospho-p53 positive tumours showed a high proliferation index compared to phospho-p53 negative tumours. A similar finding was also reported by Matsumoto et al (2004) where Serine 392 phosphorylation of TP53 was significantly associated with proliferative activity of tumour cells (Matsumoto, Furihata et al. 2004). Moreover phosphorylation of mutant TP53 at serine 392 was shown in urinary tract tumours to be associated with dominant-negative effects and thereby have pro-oncogenic functions (Furihata, Kurabayashl et al. 2002).

The HDM2 proto-oncogene binds to TP53 and acts as a negative regulator, inhibiting its transcriptional trans-activation activity Over-expression of HDM2 may overcome wild-type p53-mediated suppression of transformed cell growth, and it is one of the mechanisms inactivating TP53 function. In this study, HDM2 was positive in 23.6% of

cases. HDM2 over-expression was found more frequently in TCR probably due to the different cell population in this subgroup.

Strong positive correlation was found between p21 and HDM2 protein expression ($r=0.3337$, $p<0.005$). This could be explained by the fact that both proteins are direct transcriptional targets of TP53 and it seems that in this cohort, HDM2 has been acting more like a target than a negative regulator of TP53. A similar finding of the lack of correlation was previuosly reported in different types of cancers such as colorectal adenomas and carcinomas (Hao, Gunther et al. 1998; Abdel-Fattah, Yoffe et al. 2000)

# Chapter 4

# Molecular Analysis of *TP53*

# 4 : Molecular and Immunhistochemical Analysis of *TP53*

## *4.1 Introduction*

The Tumour protein 53 (TP53) can be thought of as a critical 'node' of the cellular circuitry. It is where various signalling pathways converge, from normal operations, like response to growth factors, to abnormal oncogenic stimuli. The activity of TP53 as a transcription factor is highly regulated by post-translational modifications, such as acetylation, phosphorylation, protein–protein interactions and protein stabilization (Batta and Kundu 2007). The regulation of protein stabilization is most critical upon the first indication of genotoxic stress. Cellular mechanisms for the rapid accumulation, stabilization and deployment of TP53 as a potent transcription factor are imperative for preventing the growth of a damaged cell. As soon as it is sufficiently stabilized, TP53 has several options at its disposal, and it is becoming more apparent that additional factors help TP53 to choose a particular cell response.

The importance of *TP53* as a tumour suppressor is reflected by its high rate of mutation in human cancer, with more than 50% of adult human tumours bearing inactivating mutations or deletions in the *TP53* gene. Mutations of *TP53* are less frequent in haematological malignancies than in solid tumours, accounting for 5% of myelodyplastic syndromes, 15% of chronic lymphocytic leukaemias, 30% of Burkitt's lymphomas and 50% of acute lymphoblastic leukaemias (Ichikawa 2000; Sahu and Das 2002; Klumb, Hassan et al. 2004).

In B-cell lymphomas, mutations are found in 10-20% of tumours. Exons 5-9 of *TP53* containing highly conserved domains have been recognized as mutation hotspots. However, mutations of *TP53* outside hotspot exons have been reported to account for 5% of all mutations (Pignon, Vinatier et al. 1994). Several studies have also associated *TP53*

mutations with poor prognosis, drug resistance and occurrence of relapses (Stokke, Galteland et al. 2000).

Involvement of TP53 mutants in cancer progression is associated with either trans-dominant suppression of wild-type p53 or a wild-type p53-independent oncogenic gain of function. Mutant TP53 can produce this trans-dominant effect via heteromerization with WT p53 in Tetramerization region (residues 319–360). Although still controversial, mutant TP53 can have a gain of function, acting as a dominant negative for TP53 family members; p73 and p63 (Marin, Jost et al. 2000). On the other hand, it has been suggested that the gain of function of TP53 mutants originate from trans-activating certain genes involved in carcinogenesis like MDR-1 (Lin, Teresky et al. 1995), c-myc, the proliferating cell nuclear antigen promoter (PCNA), the interleukin-6 promoter and the human heat shock protein 70 promoter (Tokalov, Pieck et al. 2007).

Molecular epidemiological data shows that mutations at R175, R248, R249, R273, R282, and G245 are the most common missense mutations of *TP53* (Hernandez-Boussard, Rodriguez-Tome et al. 1999). Overall, the commonest is R273, while in leukaemia and lymphomas is R248. The type of mutatgensis plays also a role in selecting the mutant formed, as shown by the in vitro role of aflatoxin B1 in causing G>A mutation in codon 249 (Lee, Lee et al. 2000).

## *4.2 Aims:*

The TP53 protein expression is relatively high in Lymphomas compared to *TP53* gene mutations. The aim of this chapter was to identify *TP53* mutations in DLBCL and correlate with the immunophenotypic markers that characterise DLBCL including TP53 and p21.

## *4.3 Objectives:*

- To assess the frequency, location and characteristic functional significance of *TP53* mutations in a cohort of DLBCL. PCR-SSCP analysis of exons 5 -9, was performed on DNA extracted from formalin fixed tissue section by micro-dissection and confirmed by sequencing.
- To correlate the mutation results with TP53 and its related proteins expression as well as the immunophenotypic markers used for routine diagnosis of DLBCL.

## *4.4 Results:*

### 4.4.1 Optimisation of Techniques:

#### *4.4.1.1 DNA extraction form fixed tissues:*

DNA extraction from formalin-fixed paraffin embedded sections has been validated in several reports. However the quality of extracted DNA has always been problematic (Soong and Iacopetta 1997). DNA, micro-dissected from tumour sections, was first extracted using phenol: Chloroform: IAA and precipitated with ethanol. Although the DNA yield was satisfactory, this technique showed a compromise in the DNA quality and was subsequently replaced by the Qiagen extraction kit. The latter showed better consistency in the PCR results. The time of proteinase K digestion was increased from 48 hours to 4 days to increase the yield as shown in Figure 4-1.

#### *4.4.1.2 Polymerase Chain Reaction:*

After optimisation of the DNA extraction method, the DNA from cases and cell line controls were amplified using Perkin Elmer thermal cycler with *TP53* exons 5-9 primer sets. Different annealing temperatures and numbers of cycles were adjusted for each primer set and the products were run on 3% agrose gel. A cell line positive control and water blank were always included in the run.

SSCP technique was subjected to further optimisation regarding the amount of PCR products used and also the gel running conditions. The running conditions are critical to nature of separation of ssDNA. The composition of MDE gel used, length of run and the current applied were all optimised to get the best separation as shown in Table 4-1. After PCR amplification, the products were run on 3% agarose gels. Although strong bands were seen for the amplified DNA extracted using phenol: Chloroform: IAA, the silver stained bands were fuzzy. Therefore the Qiagen kit was used for the extraction of all cases, which was attributed to better SSCP staining. When, strong bands were achieved, only 3µl were used in the SSCP. On the other hand, up to 8.5 µl were used for weak bands.

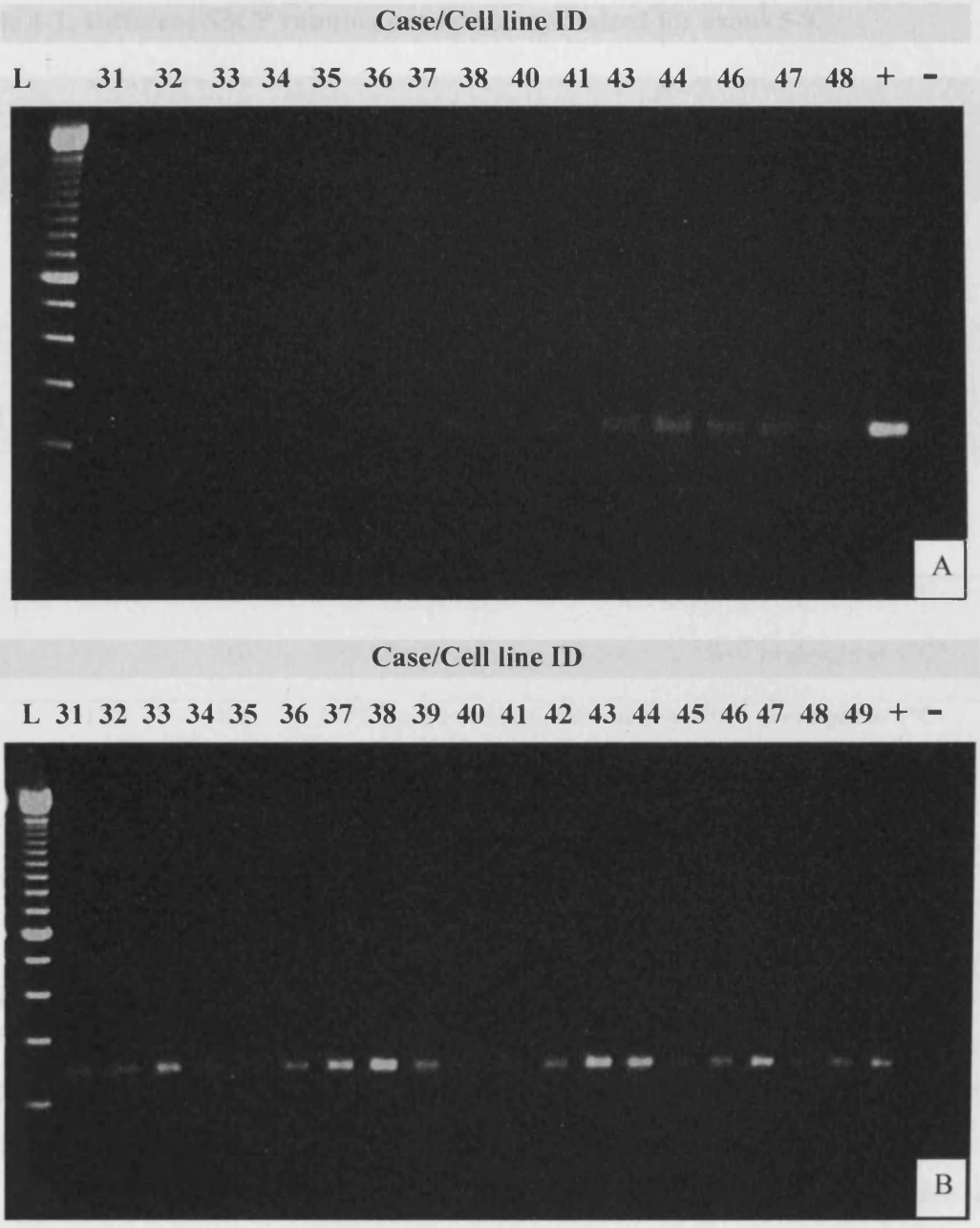



**Figure 4-1: Showing PCR products for exon 5 primer set before (A) and after (B) optimisation of the PCR conditions.** (A): Proteinase K digestion was applied for 48 hours. (B): Proteinase K digestion was applied for 4 days. Numbers represent cases ID, +:cell line control,-: water blank, L: 100bp ladder.

**Table 4-1: Different SSCP running conditions optimized for exons 5-9.**

| *Exon* | *Expected size(bp)* | *MDE gel X* | *Gel running conditions* |
|---|---|---|---|
| **5** | 245 | 0.6 | 350V for 30 minutes, followed by 300V overnight at 4°C |
| | | 0.8 | 450V for 30 minutes, followed by 300V overnight at 4°C* |
| **6** | 160 | 0.6 | 300V for 30 minutes, followed by 200V overnight at 4°C |
| | | 0.8 | 350V for 30 minutes, followed by 250V overnight at 4°C* |
| **7** | 165 | 0.6 | 300V for 30 minutes, followed by 200V overnight at 4°C |
| | | 0.8 | 350V for 30 minutes, followed by 250V overnight at 4°C* |
| **8** | 199 | 0.6 | 350V for 30 minutes, followed by 300V overnight at 4°C |
| | | 0.8 | 350V for 30 minutes, followed by 300V overnight at 4°C* |
| **9** | 127 | 0.6 | 350V for 30 minutes, followed by 300V overnight at 4°C |
| | | 0.8 | 300V overnight at 4°C* |

*Refers to the optimised conditions used for each primer set.

#### *4.4.1.3 Re-amplification of aberrant bands:*

The DNA scratched from the silver stained gel was re-amplified using the same primer set used in the original PCR. The number of cycles was increased to 40 to compensate for the small amount of starting template. Examples of the results of amplification for exons 5-9 are shown in Figures 4-2. All aberrant bands were subjected to re-amplification and sequencing in 3 different experiments. The sequencing was performed by the protein and nucleic acid chemistry laboratory (PNACL) at the University of Leicester. The approach for the selection and subsequent re-amplification of aberrant bands was described in Chapter 2.

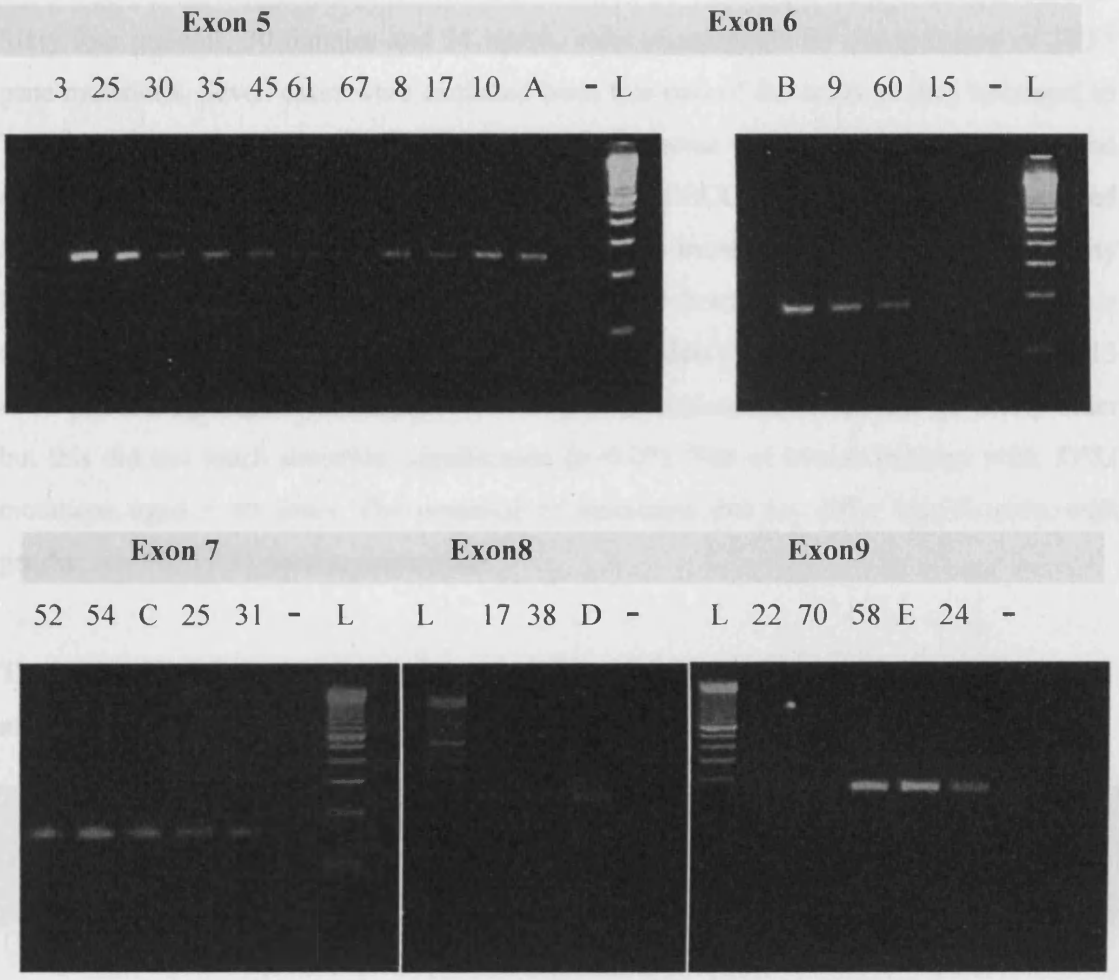



**Figure 4-2: Re-amplification of aberrant bands for 40 cycles using *TP53* exons 5-9 primers sets.** Numbers represent cases ID, A: HCT-15, B: Jurkat, C: Raji, D: HT-29, E: SW-480, L: 100 bp ladder, -: water blank.

### 4.4.2 Characteristics of the patients:

Sixty four patients, 30 females and 34 males, were investigated for the presence of *TP53* gene mutations. Seven cases were excluded from this part of the study as they belonged to TCR lymphomas where most of the DNA to be extracted would represent the T-cell and not the malignant B-cells population. One case of DLBCL failed to show any amplified PCR products on the agarose gel performed, which is most probably due to DNA quality from the fixative used. The age of the patients ranged from 19-89 at diagnosis. Patients ≥ 60 years had higher frequency of TP53 protein expression (25 of 38 cases) compared to 13 of 38 patients aged ≤ 60 years. Patients with *TP53* mutations detected were generally older but this did not reach statistical significance (p=0.07). Ten of twelve patients with *TP53* mutations aged ≥ 60 years. The presence of mutations did not differ significantly with gender, nor did TP53 protein expression.

**Table 4-2: Correlation between age ofthe patients and TP53 gene status and protein analysis.**

| | | *Age* | | *Total* | *p* |
|---|---|---|---|---|---|
| | | *<60 years* | *>=60 years* | | |
| TP53 Mutation | WTp53 | 23 | 29 | 52 | 0.07 |
| | TP53 mutation | 2 | 10 | 12 | |
| Total | | 25 | 39 | 64 | |
| TP53 IHC | Positive | 9 | 22 | 31 | 0.08 |
| | Negative | 16 | 17 | 33 | |
| Total | | 25 | 39 | 64 | |

### 4.4.3 PCR-SSCP Analysis

Several attempts had been made to optimise DNA extraction, PCR conditions and SSCP running conditions. Five cell lines were included along with cases of DLBCL. Each case was subjected to PCR amplification as mentioned in Chapter 2. No successful PCR amplification was retrieved from case 10 in all exon 5-9 primer set. This could be attributed to improper DNA quality from formalin cross-linking effect. The SSCP gel was run according to the size of amplicon produced by each primer set as mentioned before.The PCR products were tested on 3% agarose gel prior to SSCP. The amount of PCR products used in SSCP analysis was adjusted (3-8.5μl) according to the band strength.

All aberrant bands on SSCP gels were subjected to further sequencing. Cases and cell line controls were run on 0.6X MDE buffer. The gels were then silver stained and scanned. Sequencing of aberrant bands was done in triplicate for each band, and information about mutations found were obtained using p53 knowledge database (http://p53.bii.a-star.edu.sg/aboutp53/dnaseq/index.php) and IARCTP53 mutation database (http://www-p53.iarc.fr).

#### *4.4.3.1 Exon 5:*

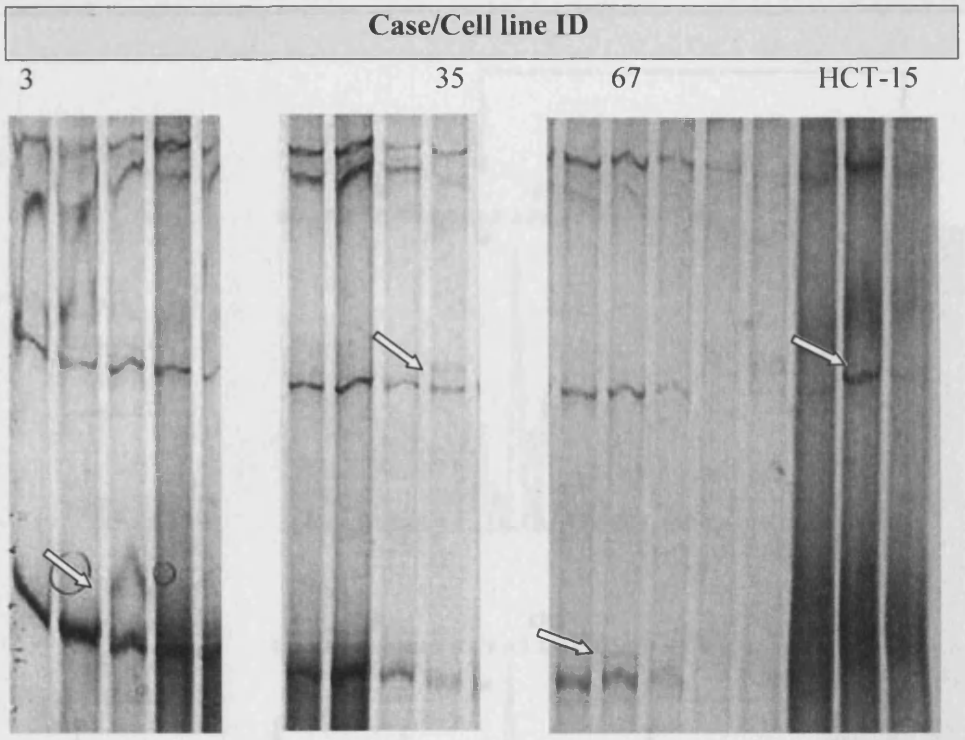



**Figure 4-3: SSCP analysis of TP53 exon 5. Arrows represent aberrant bands selected for sequencing.**

**Band A (case 3):**

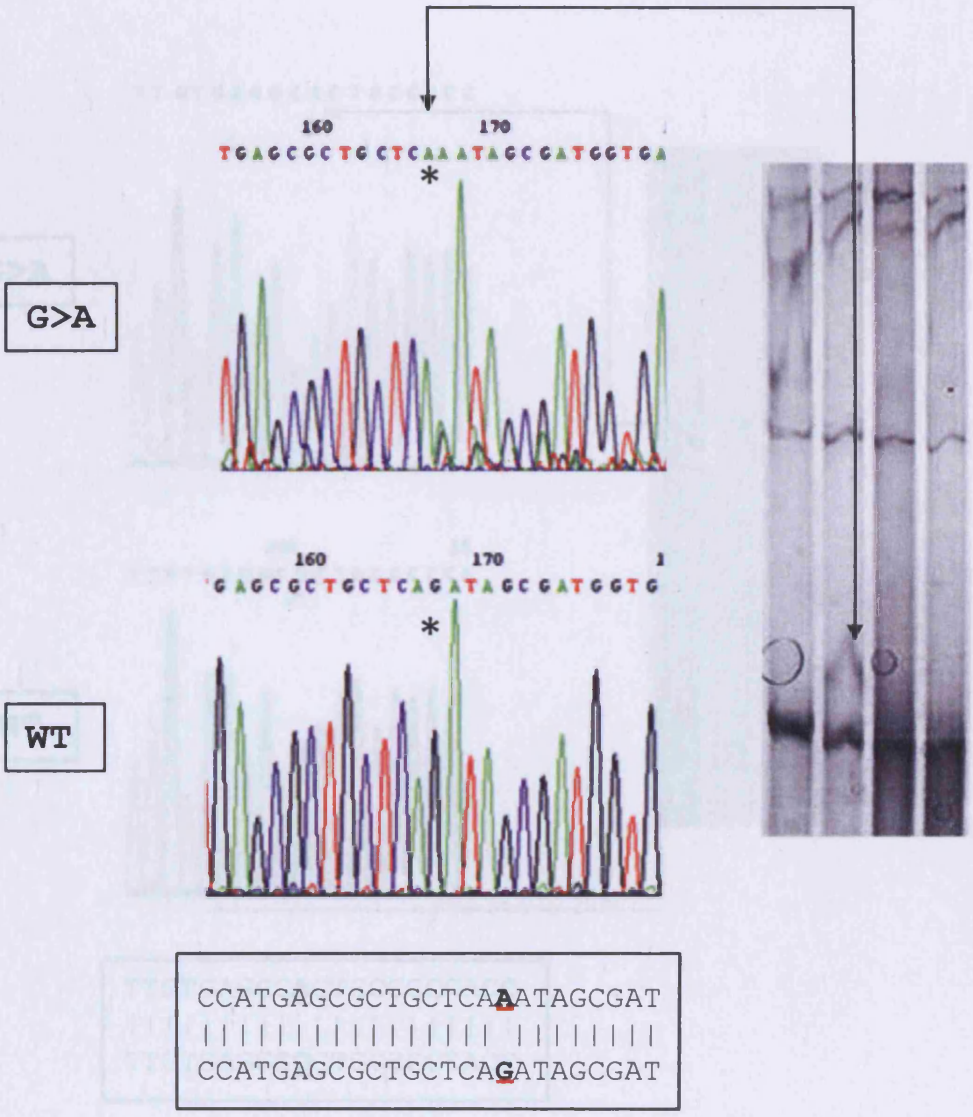



**Figure 4-4: Band A showed a G >A transition mutation at codon 184 causing an Aspartic to Aspartine amino acid change.**

**Band B (case 35):**

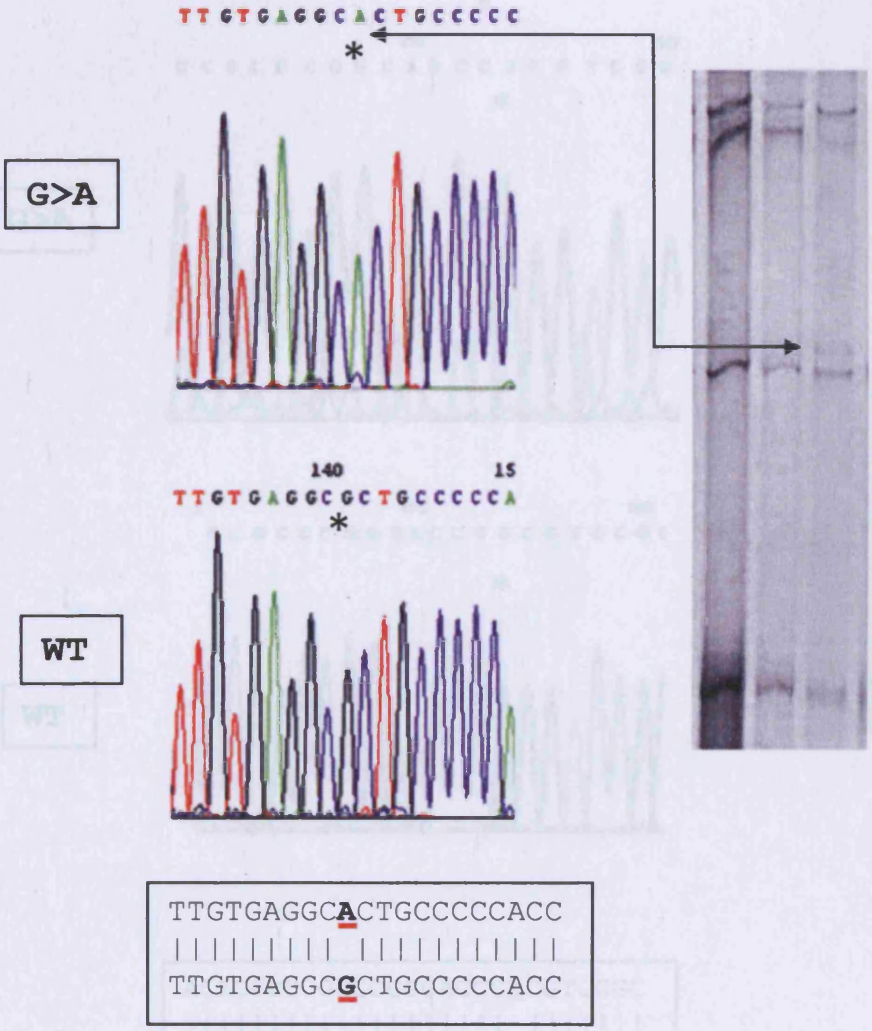



**Figure 4-5: Showing G>A transition mutation in codon 175 causing an Arginine to Histidine change.**

**Band C (case 67):**

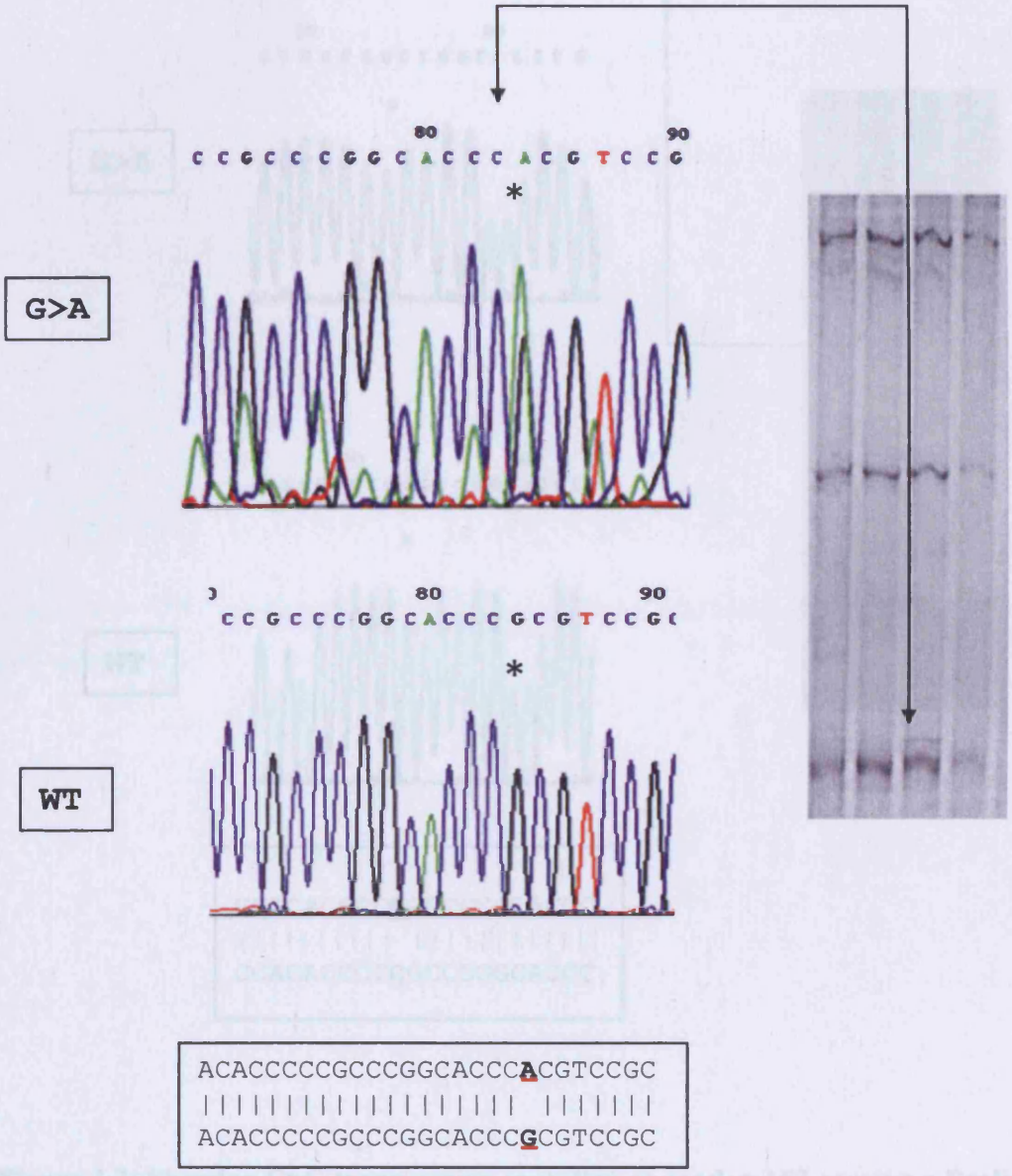



**Figure 4-6: Showing G>A transition mutation at codon 156 causing an Arginine to Histidine change.**

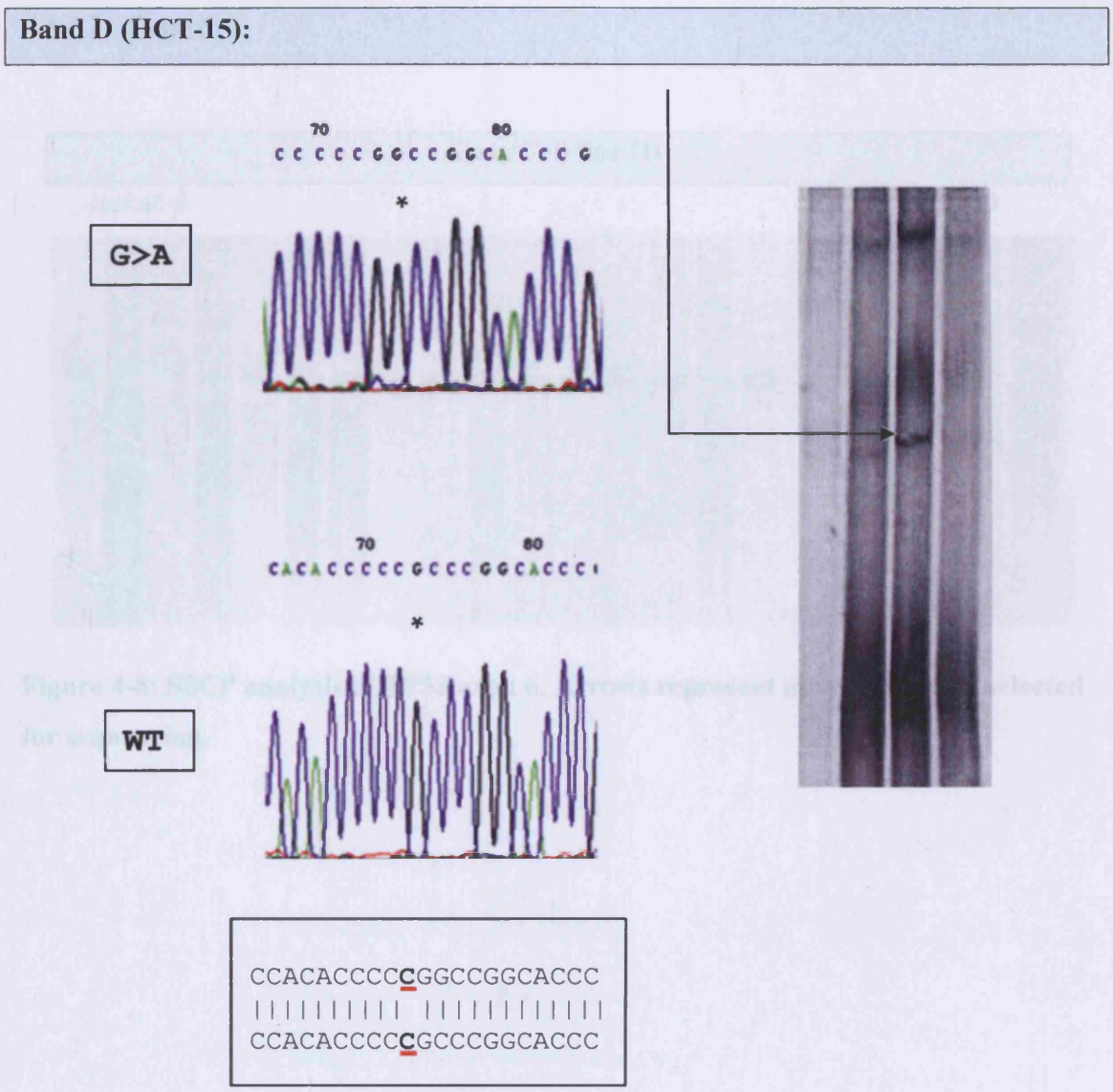



**Figure 4-7: Showing C>G transversion mutation at condon 153 causing a Proline to Serine change.**

#### *4.4.3.2 Exon 6:*

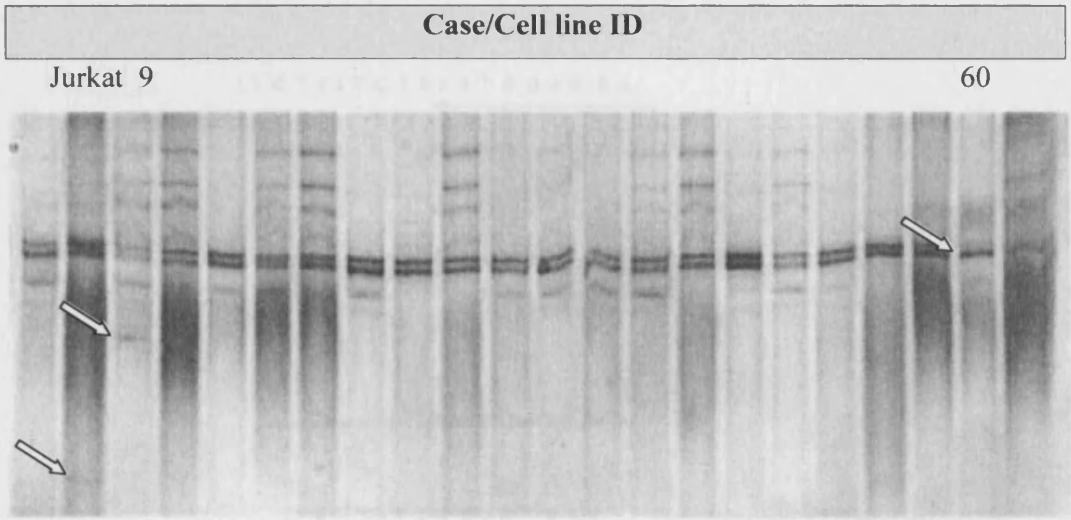



**Figure 4-8: SSCP analysis of TP53 exon 6. Arrows represent aberrant bands selected for sequencing.**

**Band E (Jurkat):**

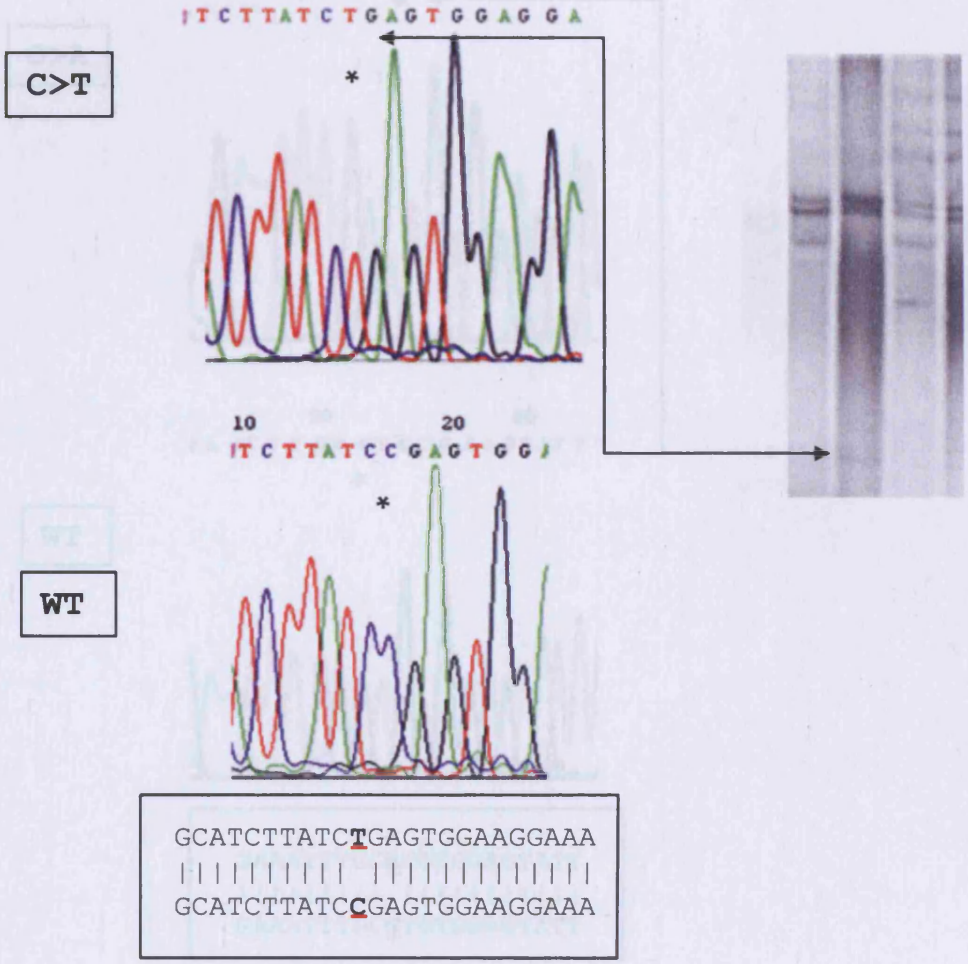



**Figure 4-9: Showing C>T mutation resulting in stop codon (196).**

**Band F (case 9):**

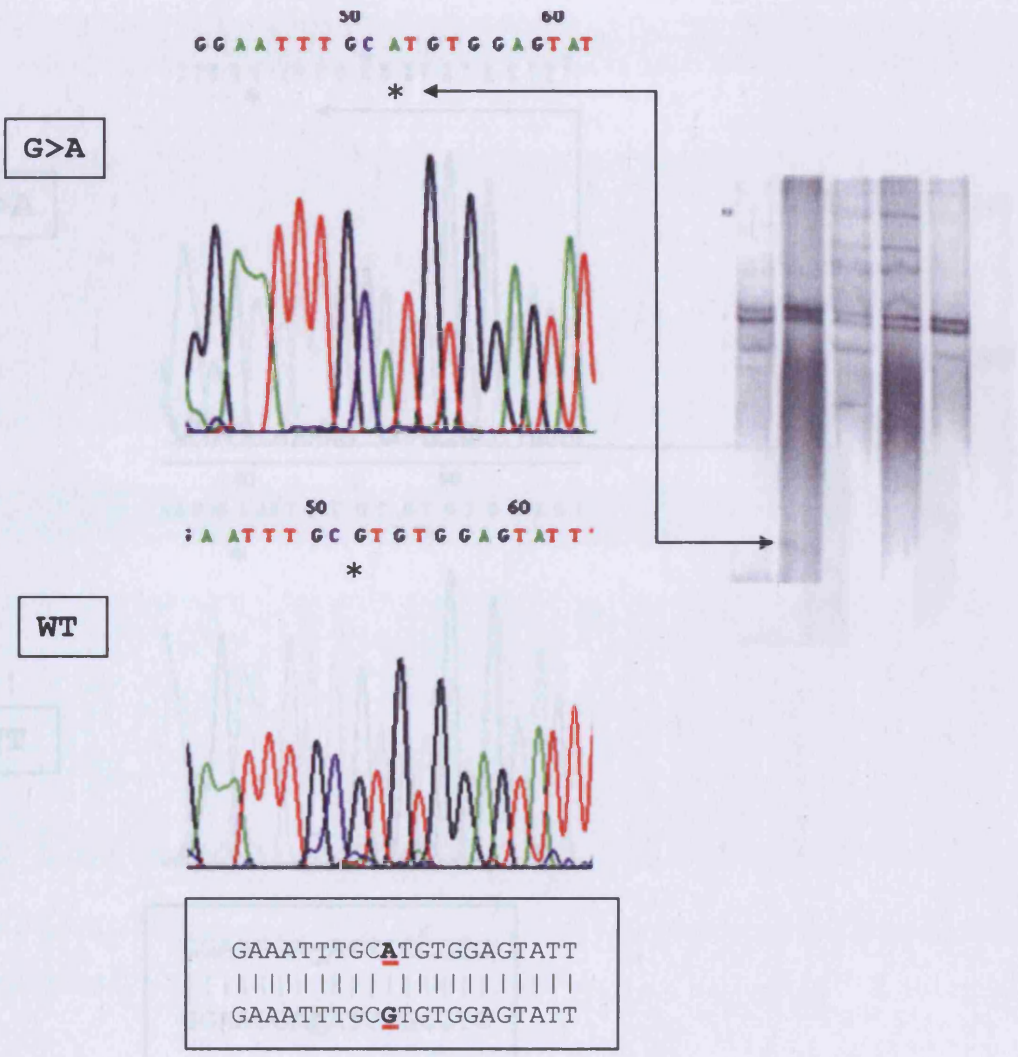



**Figure 4-10: Showing G>A transition at codon 202 causing an Arginine to Histidine change.**

**Band G (Case 60):**

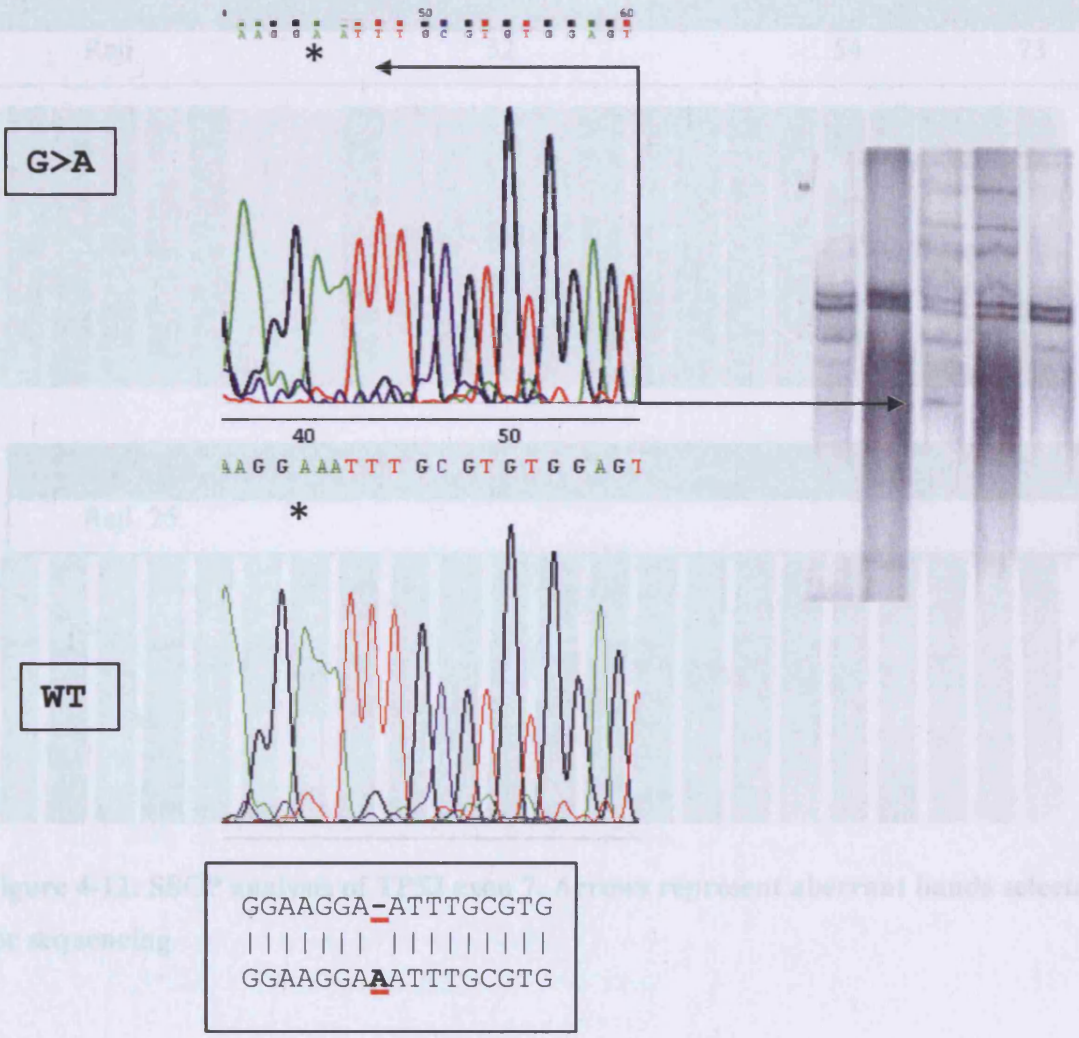



**Figure 4-11: Showing a Deletion of A at codon 200 .**

#### *4.4.3.3 Exon 7*

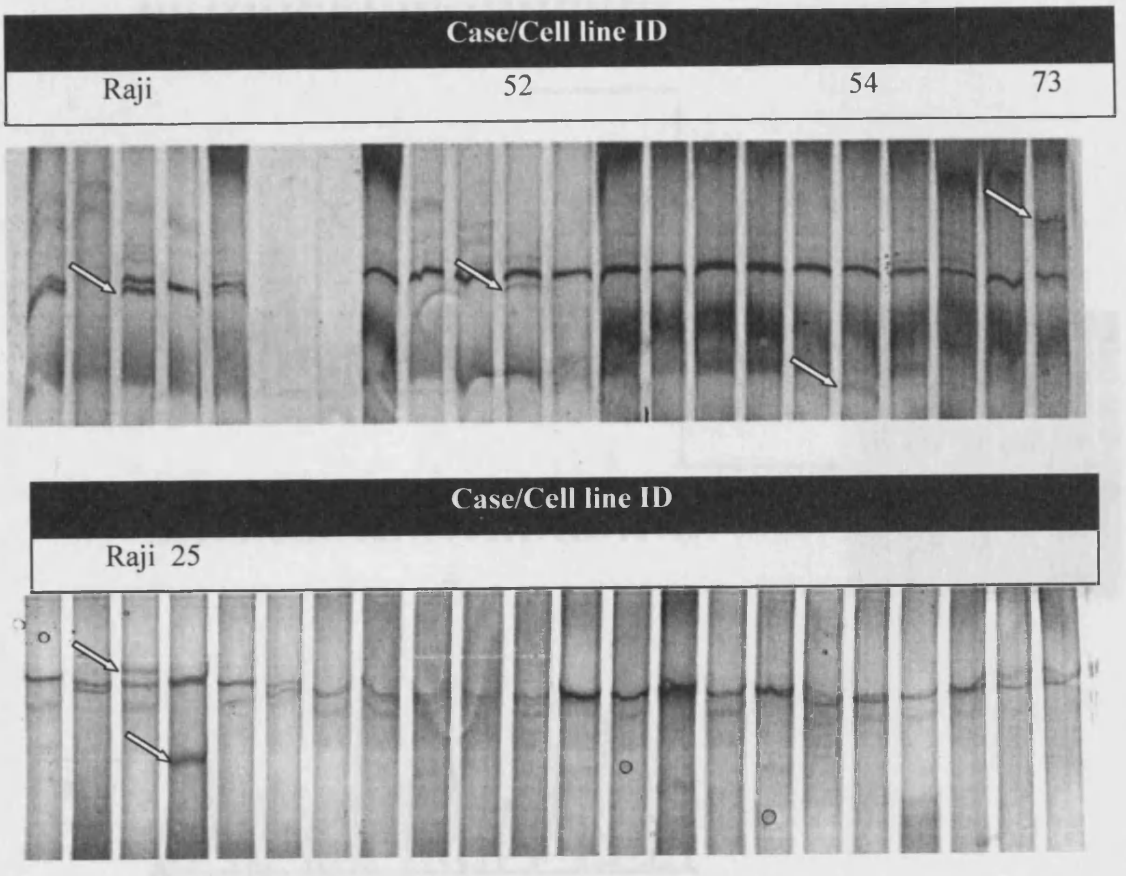



**Figure 4-12: SSCP analysis of TP53 exon 7. Arrows represent aberrant bands selected for sequencing.**

**Band H (Case 52)**

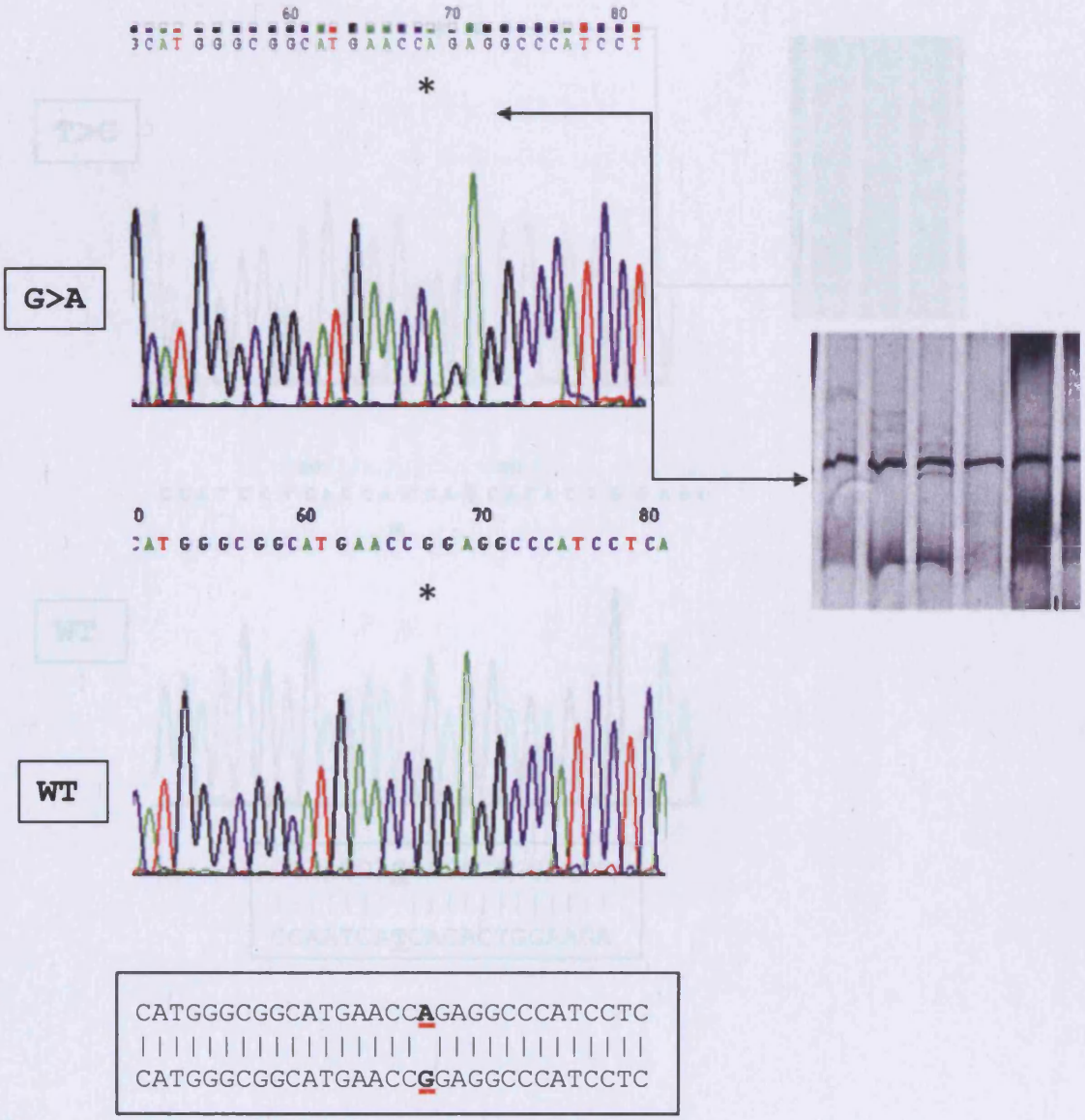



**Figure 4-13: Showing a G>A mutation at condon 248 causing an Arginine to Glutamine amino acid change.**

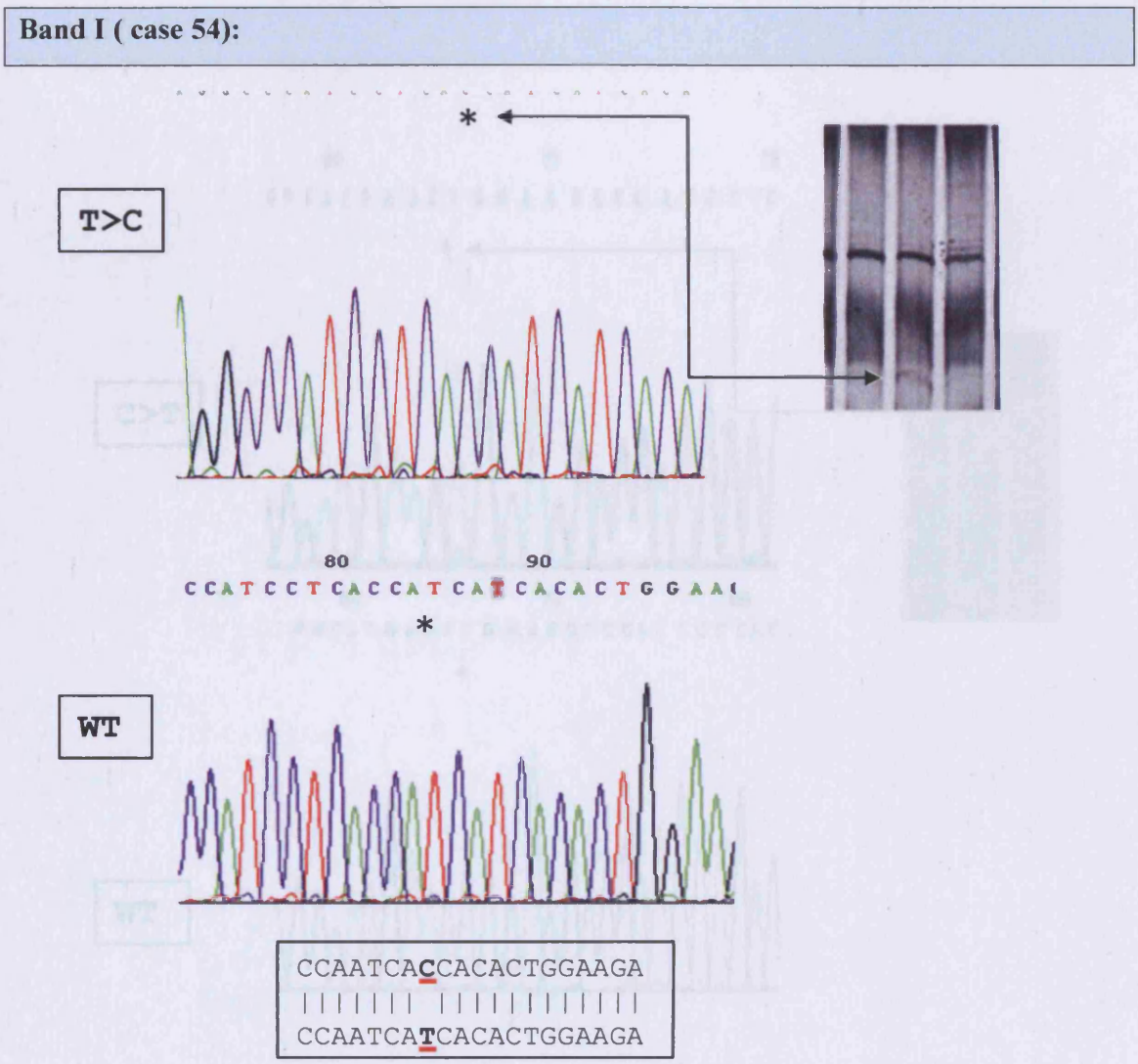



**Figure 4-14: Showing a T>C mutation at condon 255 causing an Isoleucine to Threonine amino acid change.**

**Band J (case 73):**

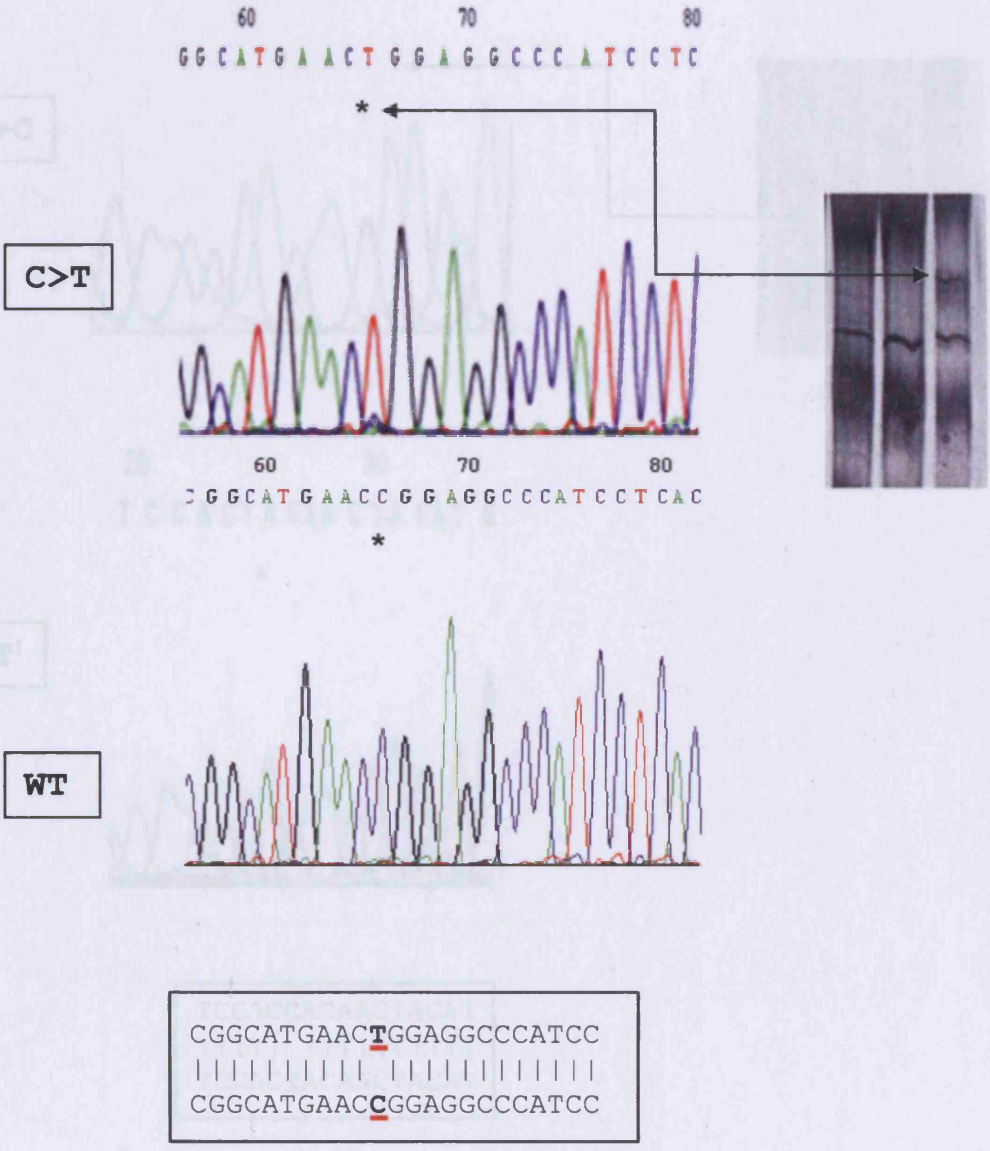



**Figure 4-15: Showing a G>A mutation at condon 248 causing an Arginine to Tryptophan amino acid change.**

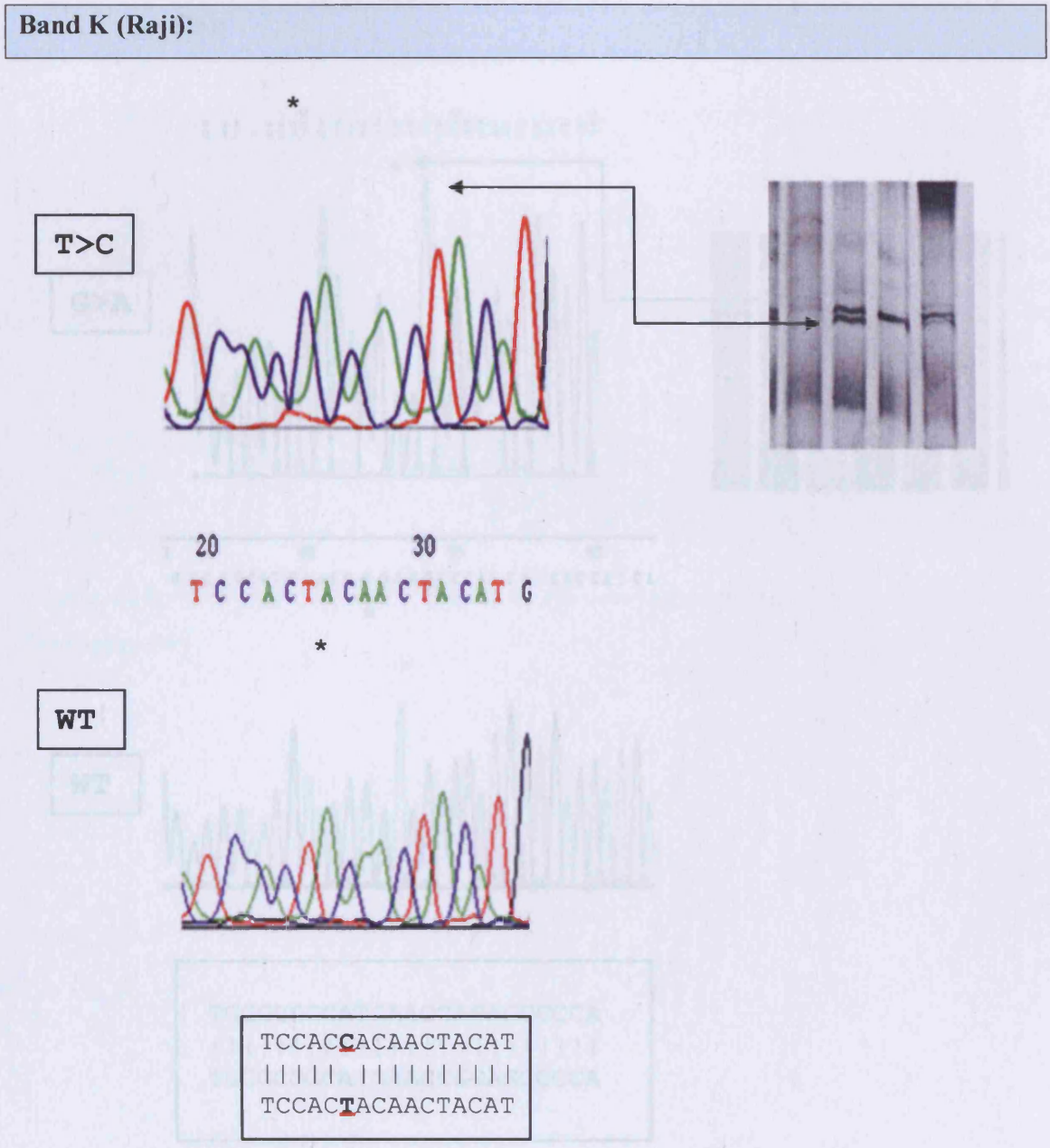



**Figure 4-16: Showing T>C transition mutation at codon 234 causing a Tyrosine to Histidine amino acid change.**

**Band L (Case25):**

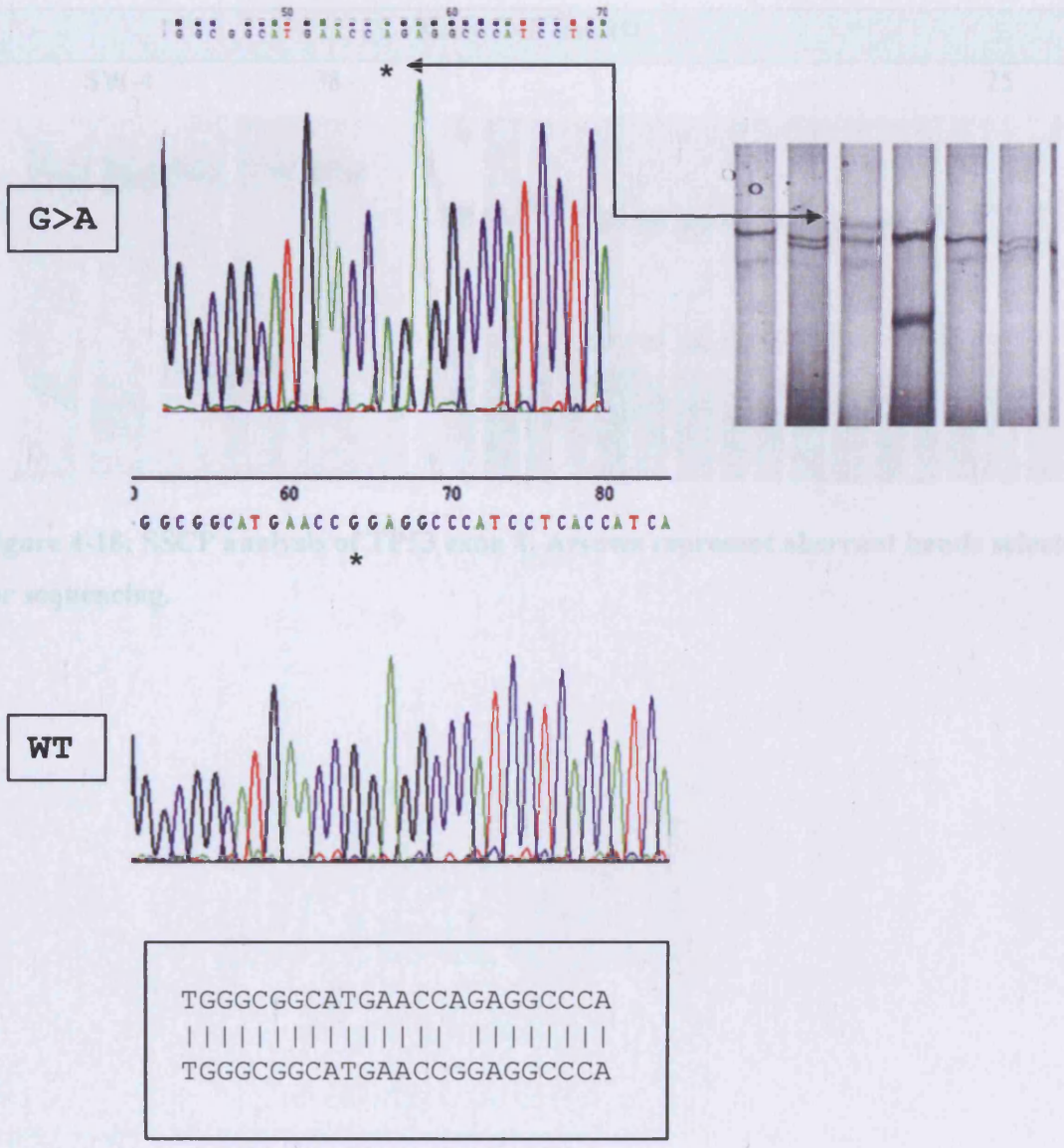



**Figure 4-17: Showing G>A transition mutation at codon 248 causing an Arginine to Glutamine AA change.**

#### *4.4.3.4 Exon 8:*

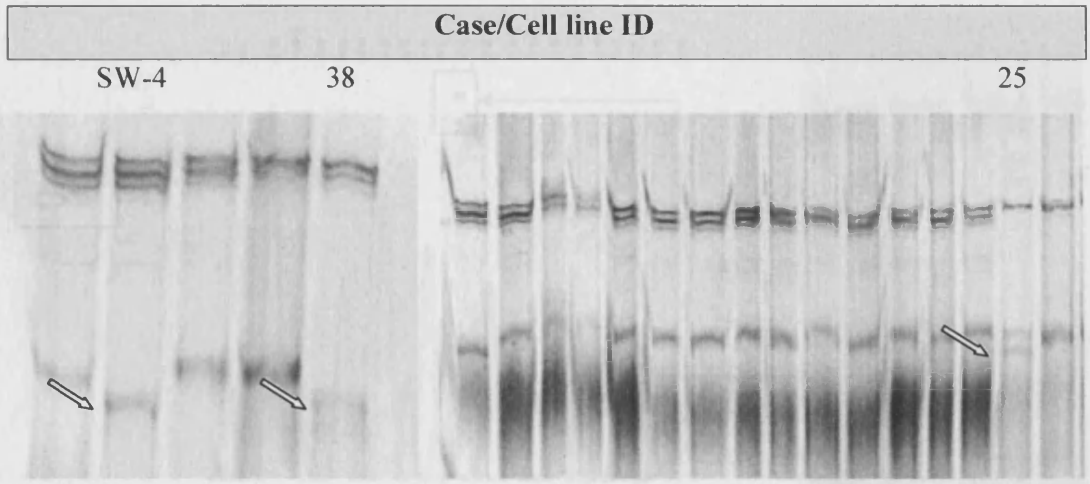



**Figure 4-18: SSCP analysis of TP53 exon 8. Arrows represent aberrant bands selected for sequencing.**

**Band M (case 17):**

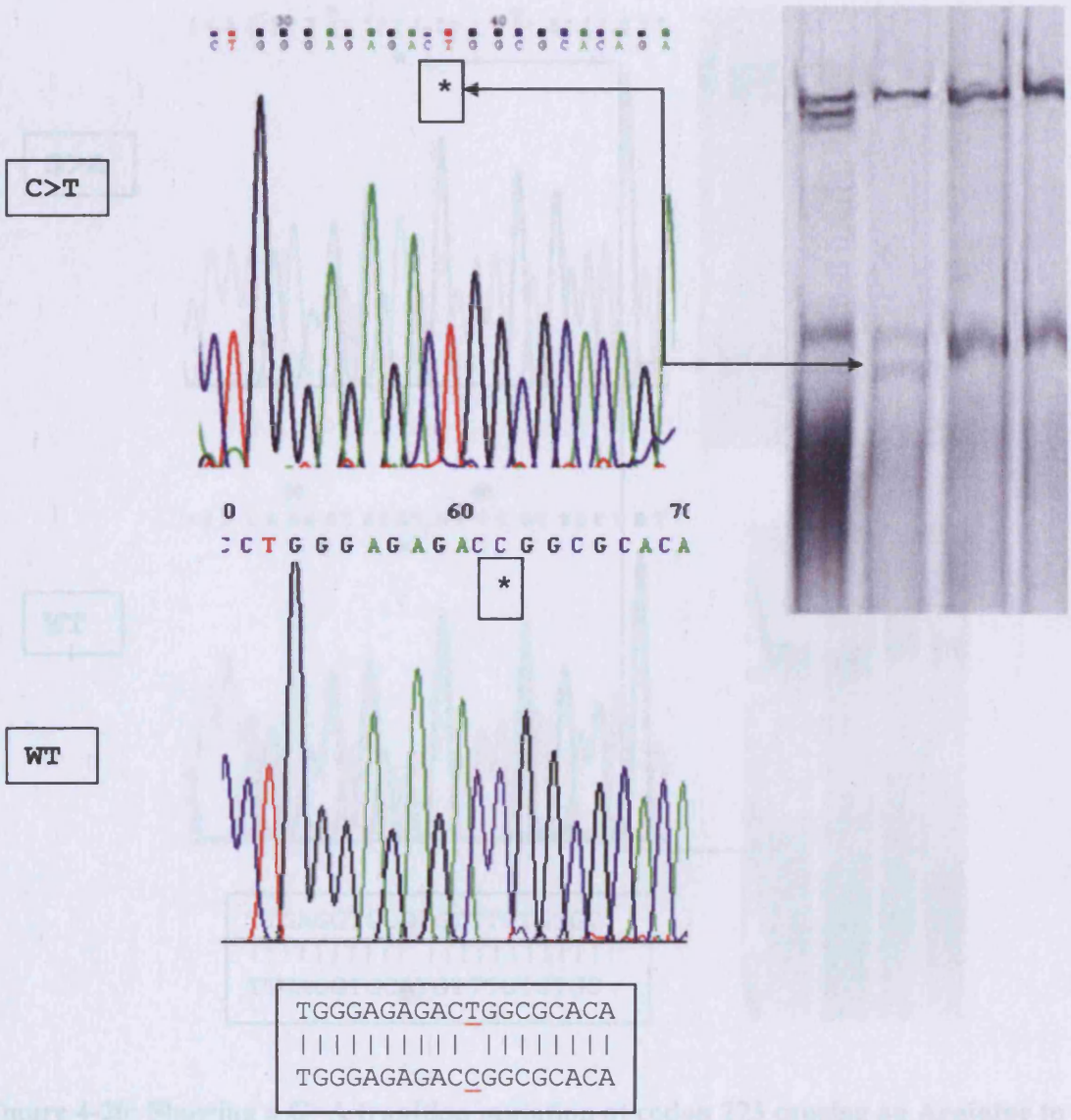



**Figure 4-19: Showing a C>T transition mutation at codon 282 causing an Arginine to Tryptophan Amino acid change.**

**Band N (case 38, HT-29 and SW-480):**

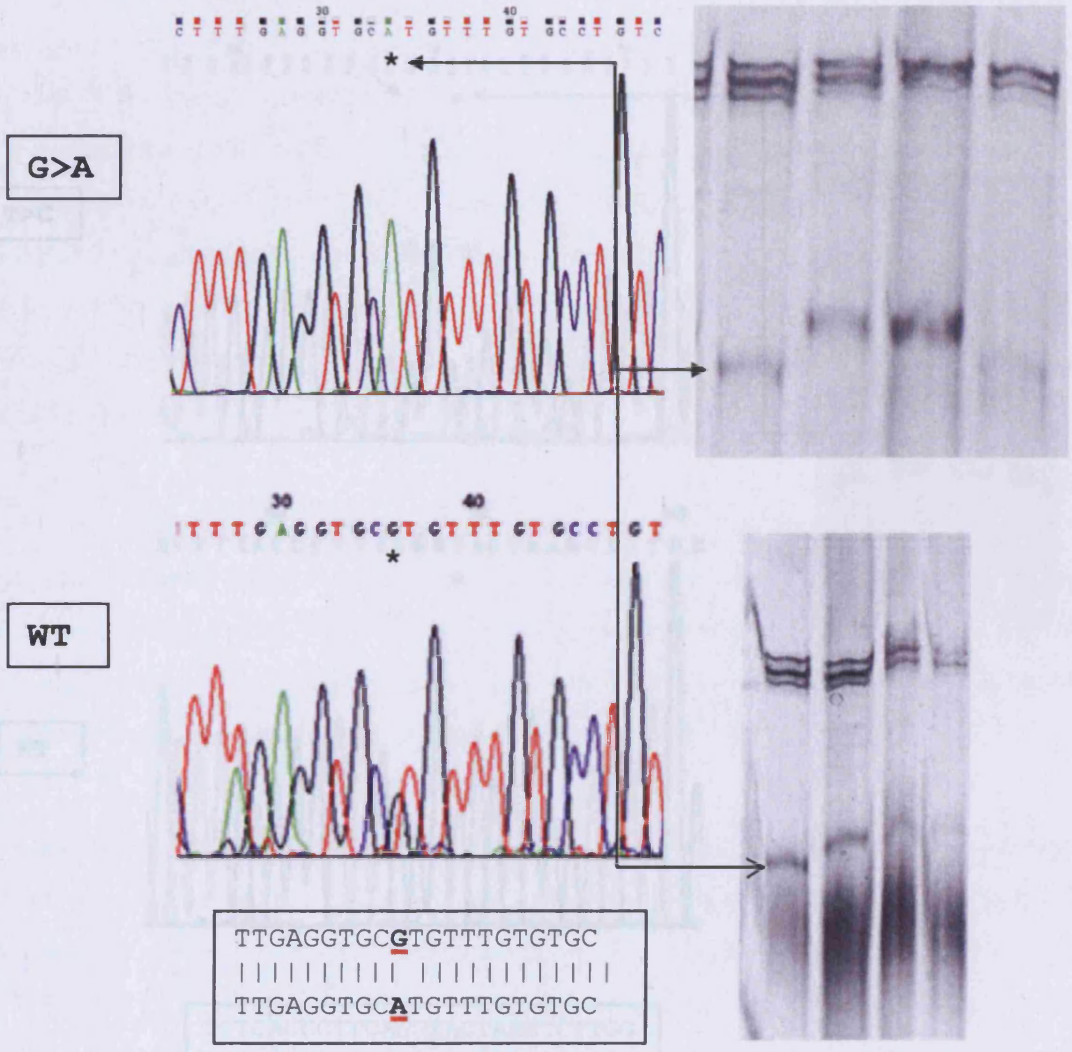



**Figure 4-20: Showing a G>A tranition mutation at codon 273 causing an Arginine to Histidine AA change.**

#### *4.4.3.5 Exon 9:*

**Band O (Case 58):**

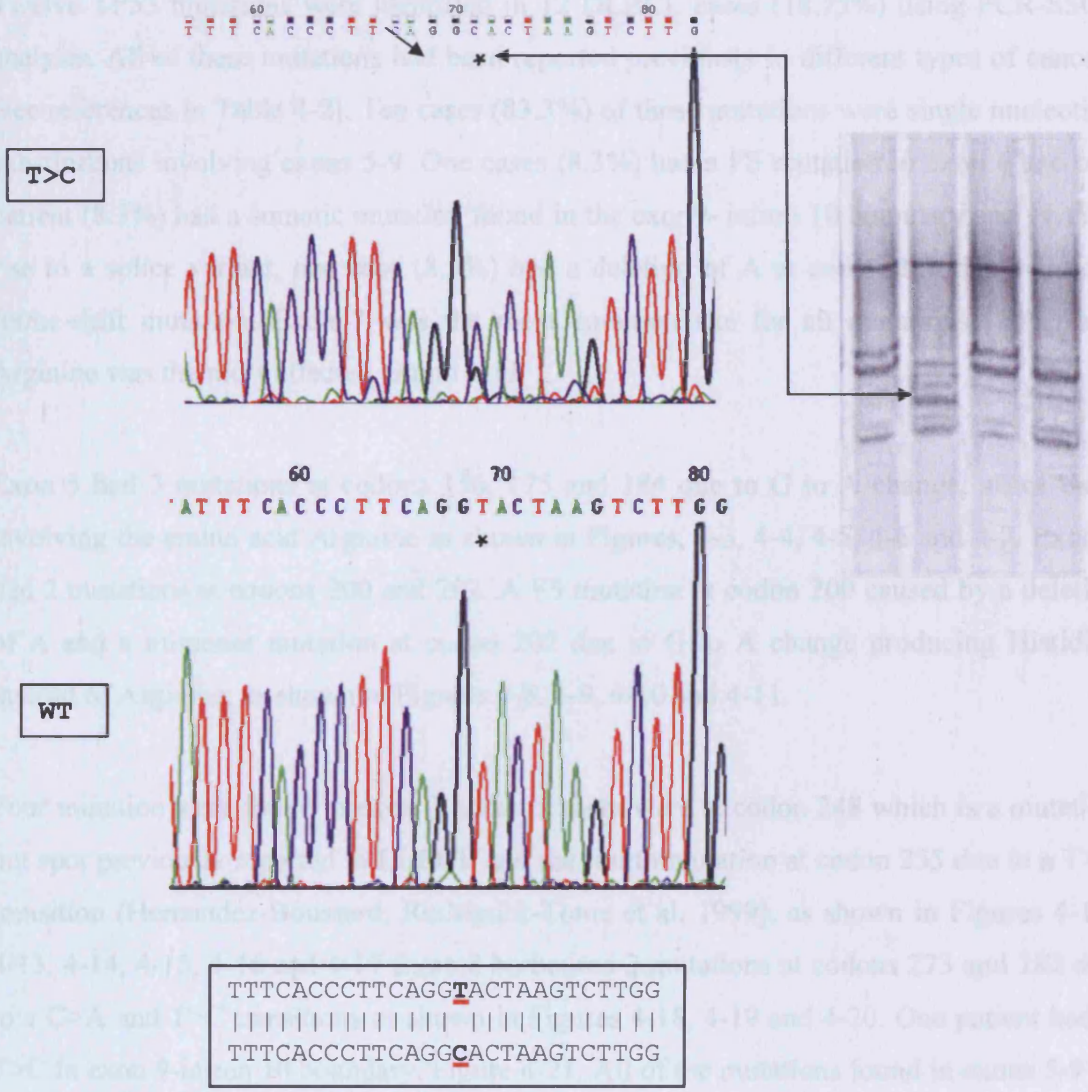



**Figure 4-21: Showing a T>C mutation in the exon 9-intron 10 boundary.**

### 4.4.4 Summary of TP53 Gene Mutations:

In this part of the study, sixty-four cases belonging to classic DLBCL were investigated. Twelve TP53 mutations were identified in 12 DLBCL cases (18.75%) using PCR-SSCP analysis. All of these mutations had been reported previously in different types of cancers (see references in Table 4-2). Ten cases (83.3%) of these mutations were single nucleotide substitutions involving exons 5-9. One cases (8.3%) had a FS mutation in exon 6 and one patient (8.3%) had a somatic mutation found in the exon9- intron 10 boundary and giveing rise to a splice variant, one case (8.3%) had a deletion of A at codon 200 that caused a frame-shift mutation. Exon 7 was the most common site for all mutations (30%) and Arginine was the most affected amino acid.

Exon 5 had 3 mutations at codons 156, 175 and 184 due to G to A change, all of them involving the amino acid Arginine as shown in Figures, 4-3, 4-4, 4-5, 4-6 and 4-7. Exon 6 had 2 mutations at codons 200 and 202. A FS mutation at codon 200 caused by a deletion of A and a missense mutation at codon 202 due to G to A change producing Histidine instead of Arginine, as shown in Figures 4-8, 4-9, 4-10 and 4-11.

Four mutation were found in exon 7, three of them were at codon 248 which is a mutation hot spot previously reported in DLBCL and the fourth mutation at codon 255 due to a T>C transition (Hernandez-Boussard, Rodriguez-Tome et al. 1999), as shown in Figures 4-12, 4-13, 4-14, 4-15, 4-16 and 4-17 Exon 8 harboured 2 mutations at codons 273 and 282 due to a G>A and T>C transitions as shown in Figures 4-18, 4-19 and 4-20. One patient had a T>C in exon 9-intron 10 boundary, Figure 4-21. All of the mutations found in exons 5-9 in cases and cell line controls are given in Table 4-2.

| | *Band* | *Type* | *Codon* | *Nucleotide change* | *Amino Acid* | *Mutant codon* | *Function* | *Structural motif* | *Reference* |
|---|---|---|---|---|---|---|---|---|---|
| **Exon 5** | A (Case 3) | Missense | 184 | G>A | Asp>Asn | AAT | PF | L2 | (Kelsey, Hirao et al. 2005) |
| | B (Case 35) | Missense | 156 | G>A | Arg>His | CAC | PF | S4 | (Walsh, Casadei et al. 2006) |
| | C (Case 67) | Missense | 175 | G>A | Arg > His | CAC | NF | L2 | (Quesnel, Verselis et al. 1999) |
| | D (HCT-15) | Missense | 153 | C>G | Pro >Ser | GCC | PF | L | (Goan, Chang et al. 2005) |
| **Exon 6** | E (Jurkat) | Stop | 196 | C>T | Arg>NA | TGA | NA | NA | (Iinuma, Okinaga et al. 2000) |
| | F (Case 9) | Missense | 202 | G>A | Arg>His | CAT | PF | L | (Feng, Shiozawa et al. 2005) |
| | G (Case60) | FS | 200 | G>A | Arg> His | -AT | NA | NA | (Tannapfel, Busse et al. 2001) |
| **Exon 7** | H (Case52) | Missense | 248 | G>A | Arg>Gln | CAG | NF | L3 | (Bendig, Mohr et al. 2004) |
| | I (Case54) | Missense | 255 | T>C | Ile>Thr | ACC | NF | S9 | (Dansonka-Mieszkowska, Ludwig et al. 2006) |
| | J (Case 73) | Missense | 248 | C>T | Arg>Trp | TGG | NF | L3 | (Pivnick, Furman et al. 1998) |
| | K (Raji) | Missense | 234 | T>C | Tyr>His | CAC | NF | S8 | (Gwosdz, Balz et al. 2005) |
| | L (Case 25) | Missense | 248 | G>A | Arg>Gln | CAG | NF | L3 | (Bendig, Mohr et al. 2004) |
| **Exon 8** | M (Case 17) | Missense | 282 | C>T | Arg>Trp | TGG | NF | H2 | (Bougeard, Limacher et al. 2001) |
| | N (HT-29, SW-480, Case 38) | Missense | 273 | G>A | Arg >His | CAT | NF | S10 | (Wong, Verselis et al. 2006) |
| **Exon 9** | O (Case58) | Splice | Intron 10 | T>C | NA | | NA | NA | (Ott K, (2003) |

**Table 4-3: Total *TP53* mutations identified and sequence data for cases and cell line controls.** PF: partially functional, NF: non-functional, NA: Non-available.

### 4.4.5 Correlation between TP53 gene mutations and protein expression:

Under normal conditions, WT-p53 has a half-life of 30 minutes. Unlike other tumour suppressor genes, which are usually hit by a deletion or translocations, *TP53* mutations are mostly missense. The mutation alters the TP53 conformation and leads to a stabilized protein that is readily detected by immunohistochemical methods. Alternatively, this TP53 protein over-expression could be due to the rapidly proliferating nature of high-grade lymphomas.

WT p53 was found in 32 of 33 (96.96% %) cases that were negative for TP53 by IHC, There was a strong positive correlation between TP53 negativity by immunohistochemistry and WT-p53 ($p<0.005$). On the other hand, eleven of the thirty-one cases (35.48 %) negative for DO-1 staining (<20%), had a confirmed mutation in *TP53.* There was no significant correlation between TP53 positivity and the presence of mutation ($p=0.333$). Similarly, TP53 phosphorylated at Serine 392 showed a significant correlation of its negativity by immunohistochemistry (40 of 43 cases, $p\leq0.005$) and the absence of mutation of *TP53* gene. Nevertheless, there was no correlation between its positivity and the presence of such mutation (9 of 21 cases, $p=0.591$).

**Table 4-4: Correlation of TP53 and TP53 phosphorylated at Serine 392 protein expressions and *TP53* mutational status.**

| | *TP53* | | *Phospho-p53* | | *Total* |
|---|---|---|---|---|---|
| | + | - | + | - | |
| WT-p53 | 20 | 32 ($p<0.001$) | 12 | 40 ($p<0.001$) | 52 |
| Mutant p53 | 11 ($p=0.333$) | 1 | 9 ($p=0.591$) | 3 | 12 |
| Total | 31 | 33 | 21 | 43 | 64 |

### 4.4.6 Correlation of TP53 Gene Mutations and Different TP53 Induced Proteins:

Although TP53 and phospho-p53 proteins under-expression significantly correlated with the absence of TP53 gene mutations ($p<0.05$), there was no significant correlation between TP53 gene mutations and p21 and HDM2. While taking in consideration that the TP53 protein over expression could be due to mutations of the TP53, resulting in the stabilization of mutant TP53 that could be detected by immunohistochemistry or due to over-expression of the WT-p53 in response to carcinogeneic stresses, different TP53/p21 expression patterns were further studied.

**Table 4-5: Correlation between TP53 gene status and immunohistochemical markers.**

| | *TP53* | | *Phospho-p53* | | *p21* | | *MDM2* | | *Total* |
|---|---|---|---|---|---|---|---|---|---|
| WT-p53 | + | - | + | - | + | - | + | - | |
| | 20 | 32 | 12 | 40 | 11 | 41 | 11 | 41 | 52 |
| Mutant TP53 | 11 | 1 | 9 | 3 | 1 | 11 | 1 | 11 | 12 |
| p | 0.001 | | 0.001 | | 0.436 | | 0.436 | | |

### 4.4.7 Association of T53 Gene Mutations with TP53 and p21 Expression Patterns:

p21 /WAF-1 is a universal cdk- inhibitor implicated in mediating TP53–dependent G1 growth arrest and cellular differentiation. Because mutations of p21 gene are very rare, p21 has been regarded as functional TP53 marker. To further analyze cases with TP53 over expression, p21 was included to differentiate between functional and non functional TP53.

There was a statistically significant correlation found between *TP53* mutations, regardless of its type, and TP+/p21- phenotype ($p<0.05$). With the detailed analysis according to the mutation type, TP53+/p21- phenotype was found in 10 of 10 cases with missense mutations in exons 5-8 ($p<0.005$). One case with a Frame shift mutation in exon 6 had a TP53-/p21- phenotype while a splice variant had a TP53+/p21+ corresponding to functional TP53 as shown in Figure 4-23 and Table 4-6

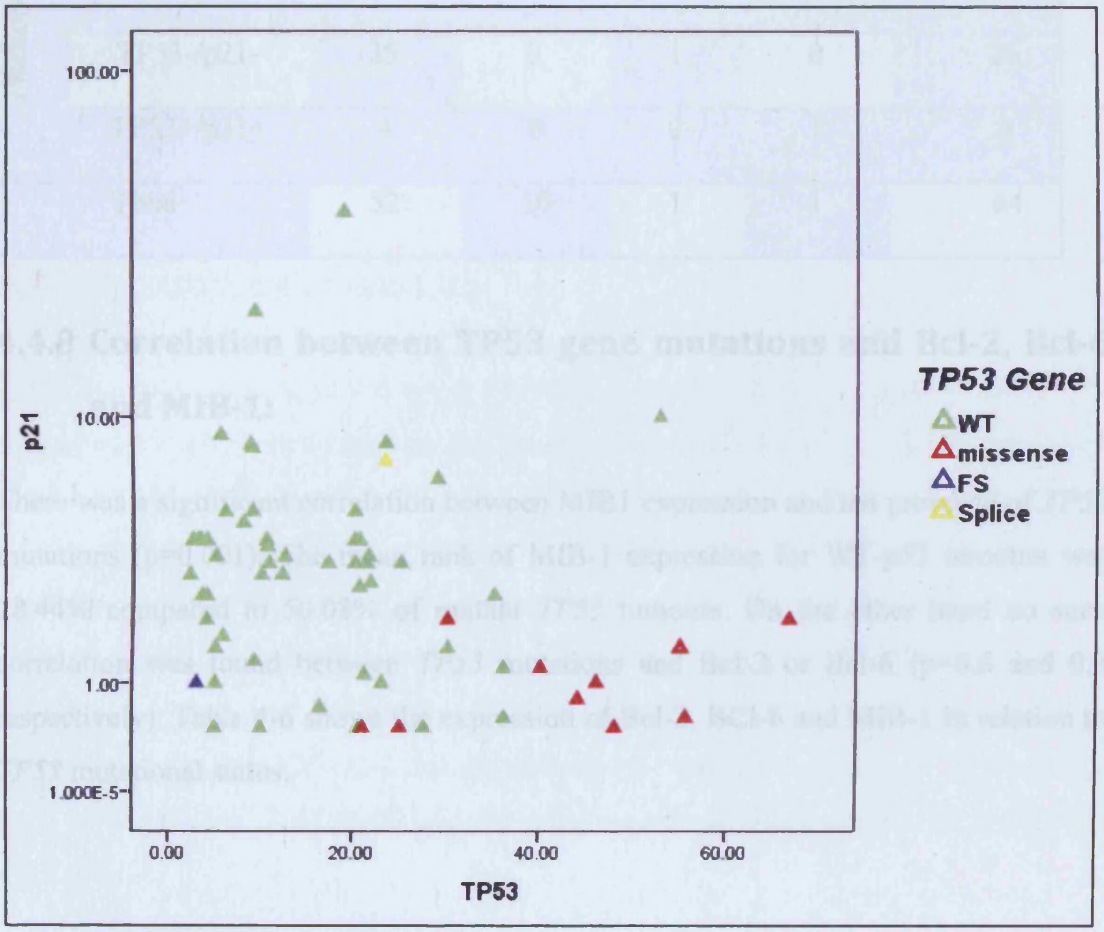



**Figure 4-22: The relationship between TP53 gene status and TP53/p21 expression patterns.** All of TP53 missense mutations have a TP53+/p21- phenoptype.

**Table 4-6: TP53 and p21 proteins in relation to *TP53* mutations.**

| | | *Mutation Type* | | | | *Total* |
|---|---|---|---|---|---|---|
| | | *WT p53* | *Missense* | *FS* | *Splice* | |
| *Phenotype* | TP53-/p21+ | 7 | 0 | 0 | 0 | 7 |
| | TP53+/p21- | 16 | 10 | 0 | 0 | 26 |
| | TP53-/p21- | 25 | 0 | 1 | 0 | 26 |
| | TP53+/p21+ | 4 | 0 | 0 | 1 | 5 |
| | Total | 52 | 10 | 1 | 1 | 64 |

### 4.4.8 Correlation between TP53 gene mutations and Bcl-2, Bcl-6 and MIB-1:

There was a significant correlation between MIB1 expression and the presence of *TP53* mutations (p=0.001). The mean rank of MIB-1 expression for WT-p53 tumours was 28.44% compared to 50.08% of mutant *TP53* tumours. On the other hand no such correlation was found between *TP53* mutations and Bcl-2 or Bcl-6 (p=0.5 and 0.3 respectively). Table 4-6 shows the expression of Bcl-2, BCl-6 and MIB-1 in relation to *TP53* mutational status.

**Table 4-7: Correlation of TP53 mutational status with MIB-1, Bcl-2 and Bcl-6 protein expression.**

| *TP53 gene* | *MIB-1 mean rank* | *Bcl-2* | | *Bcl-6* | | *Total* |
|---|---|---|---|---|---|---|
| | | - | + | - | + | |
| WT-p53 | 28.44% | 24 | 28 | 25 | 27 | 52 |
| Mutant-*TP53* | 50.08% | 6 | 6 | 4 | 8 | 12 |
| p | 0.001 | 0.081 | | 0.35 | | 64 |

## 4.4.9 Significance of the data:

TP53 mutations were assessed using PCR-SSCP analysis in 64 cases of DLBCL. The frequency of TP53 mutations in this study was 18.75%. Exon 7 harboured most mutations (30%), followed by exon 5 (25%) and exons 6 and 8 (16.7% each). There was a significant correlation between low expression of TP53 and phospho-p53 assessed by immunohistochemistry and the presence of WT-p53, but not vice versa. Also, a significant correlation was found between missense *TP53* mutations and TP53+/p21- phenotype ($p<0.005$). High proliferation index correlated with *TP53* mutations ($p<0.005$). Other imuunohistochemical markers investigated in the study such as p21, HDM2, Bcl-2 and Bcl-6 did not shown a significant correlation with *TP53* mutations.

## *4.5 Discussion*

*TP53* located on chromosome 17p13 is by far the most studied tumour suppressor gene (Miller, Mohandas et al. 1986). It encodes a 53-KD nuclear phosphoprotein that plays a crucial role on the control of cell cycle. TP53 acts a sensor for cellular stress that has many functions ranging from cell cycle arrest, DNA repair or apoptosis. *TP53* gene mutations have been associated with nearly 50% of human cancers regardless of the origin. However in lymphomas, *TP53* gene mutations are not a frequent event in the tumourigenesis , accounting for 10-20% of tumours (Peller and Rotter 2003). Mutations of *TP53* are usually associated with advanced disease, poor survival and resistance to chemotherapy. It has been also related to transformation of follicular lymphomas (Lo Coco, Gaidano et al. 1993).

Mutations of TP53 are usually missense, unlike other tumour suppressor genes. They are mostly located in exons 5 through 9 which contain the highly conserved domains. Mutational hotspots occur in codons 175, 245, 248 and 273 (Hainaut, Soussi et al. 1997). Although the presence of inactivating mutations is the main causes of loss of TP53 function, other mechanisms may also operate in tumours lacking of inactivating mutations.

Mutant TP53 has an extended half-life detected by immmunohistochemistry, which unlike TP53 mutation is quite common finding in lymphomas. In this study, TP53 protein over-expression was found in 48.4% of DLBCL. Sixty four cases were selected for SSCP and confirmed by sequencing. The present study analysed mutations of *TP53* exons 5-9 in 64 cases of classic DLBCL. This region includes the majority of *TP53* mutations reported in different types of cancers and in lymphomas in particular. In this study mutations of *TP53* were identified in 18.75%, which is within the range previously reported in other studies ranging around 20% (Sanchez-Beato, Saez et al. 2001; Kerbauy, Colleoni et al. 2004).

Mutations outside of exons 5–9 constitute less than 5% of mutations in general and are infrequent in NHL (Hoollstein M 1991). Exon 7 was the most commonly affected exon

(30%), especially at codon 248. This was also reported by other investigators (Klumb, Furtado et al. 2003). While other studies showed higher prevalence of exon 5 (Kamata, Mitani et al. 2007) or exon 6 (Bhatia, Gutierrez et al. 1992) mutations in regards to the frequency of *TP53* mutations among different exons. Other mutations found in this study are; one FS mutation in exon 6 and one splice variant in exon 9 and have been reported before (Eguchi, Yao et al. 2000; Ott, Vogelsang et al. 2003).

Concerning *TP53* alterations, most were point mutations that lead to an increase in protein stability and accumulation in the nucleus of tumour cells. An important finding that emerged from this study is TP53 expression dissociated from the presence of mutations. *TP53* gene mutations was found in 11/31cases showing TP53 protein over-expression ($p=0.333$). However, a very significant association between TP53 protein negativity and the absence of mutation ($p<0.005$) was found. One possible explanation for this apparent discrepancy is that the distribution of mutations in high-grade lymphomas is not limited to the classic hot spots of the *TP53* gene (Kocialkowski, Pezzella et al. 1995). Nevertheless, mutations in the exons 4 and 10 are rare and frequently these mutations are FS or non-sense type. Such null mutations are usually not detected by IHC analysis because no protein is produced (Soussi and Beroud 2001).

On the order hand, the specificity of SSCP analysis is <75% and some mutations may simply have escaped detection. Moreover TP53 over-expression can be detected in begnin and neoplastic cells, which are rapidly proliferating (Danova, Giordano et al. 1990) and not related to *TP53* mutations. Other mechanisms that may lead to TP53 protein accumulation could not be excluded in this study either. Such mechanisms like the binding to SV40 Large T-antigen (Amara, Trimeche et al. 2008), HPV- E6 and E7 and Ebstein Barr virus in cases of Burkitts lymphoma have been documented (Werness, Levine et al. 1990).

TP53 protein expression (≥20%) was found in 31 of 64 cases of DLBCL, after excluding T-cell rich subgroup and one case that failed to show any bands after PCR amplification. Eleven of the thirty one cases (35.5%) positive for DO-1 had a confirmed

mutation of TP53 gene. There was no significant correlation between TP53 positivity and the presence of mutation.

This has led to the proposal that TP53 detection being synonymous with *TP53* mutation is not valid in this study. Nevertheless, the findings of this series, where no mutation was detected in 20 out of 31 cases with high TP53 levels, do suggest that the nuclear or cytoplasmic stabilization of TP53 protein could also depend on other factors has been described in soft-tissue sarcomas (Oliner, Pietenpol et al. 1993). Similar finding was reported in several studies showing that TP53 expression is independent of *TP53* mutations (Oka, Sarker et al. 1998; Sanchez-Beato, Sanchez-Aguilera et al. 2003). Similarly the TP53 phosphorylated at Serine 392 showed a significant correlation of its negativity by immunohistochemistry (40/43 cases, $p \leq 0.005$) and the absence of mutation of *TP53* gene. Nevertheless, there was no correlation between its positivity and the presence of such mutation (9 of 21 cases, $p=0.591$).

There was no correlation between *TP53* mutations and p21, HDM2 or other biological parameters of the studied group, this relates to several published studies reporting the absence of correlation between TP53 mutations and p21 (Chapusot, Assem et al. 2001) which could be explained by the presence of TP53-independent mechanisms for p21 regulation (Mottet, Pirotte et al. 2009). Also the absence of correlation between TP53 mutations and age or gender have been documented in previuos studies (Huang, Jin et al. 2001).

Four patterns of TP53/p21 were established. There was statistically significant correlation found between *TP53* mutations, regardless of its type, and TP53+/p21- phenotype ($p<0.05$). with further classification of mutations according to its type, TP53+/p21- phenotype was found in 10 of 10 cases with missense mutations in exons 5-8 ($p<0.005$). A similar finding was reported by several groups (Villuendas, Pezzella et al. 1997; Girlando, Slomp et al. 1999), and was explained by the fact that missense mutations of TP53 at the core domain results in a protein with a prolonged half-life that fails to bind to DNA (Kern, Kinzler et al. 1991) and subsequently fails to up-regulate the transcription of many TP53 target genes, mainly p21 (Kern, Kinzler et al. 1991).

This finding was also confirmed by subsequent studies further suggesting that the TP53+/p21- phenotype could be used as a marker for *TP53* gene status in NHL (Went, Dellas et al. 2004).

In this study, a significant correlation was found between *TP53* mutations and high proliferation index ($p<0.005$). The mean rank of MIB-1 staining by immunohistochemistry was 28.44% in WT-p53 compared to 50.08% where *TP53* mutations were found. This in concordance with other studies, showing a similar finding, in other types of tumours such as nerve sheath and breast cancer (Kindblom, Ahlden et al. 1995; Offersen, Alsner et al. 2008).

However, no correlation was found between TP53 mutations and Bcl-6 nor with Bcl-2 ($p=0.35$ and 0.081 respectively). The relationship of TP53 and Bcl-6 is complex. First Bcl-6 is a direct transcriptional target of Tp53 and second Bcl-6 can suppress TP53 in normal GC B-cells. The presence of TP53 mutations was found equally distributed among Bcl-2 positive and negative cases (6 in each group). These results are in concordance with previous reports studying the relationship between TP53 and Bcl-2 and Bcl-6 (Amara, Trimeche et al. 2008).

# Chapter 5

# miR-34a as a new target of TP53 in DLBCL

# 5 : MiR-34a as new target of TP53 in DLBCL:

## *5.1 Introduction*

Mutations of *TP53* occur in more than half of human cancers, this had led to an extensive research on *TP53*, as the most important tumour suppressor gene over the past twenty years. TP53 leads to diverse cellular responses varying from cell cycle arrest, activation of apoptosis and DNA damage repair. Which pathway is actually activated is tightly coordinated in a context- dependent manner. The discovery of extensive network of miRNAs and up or downregulation in cancers could represent another arm in this complex pathway (He, He et al. 2007).

In theory, miRNAs might act like both tumour suppressor genes and oncogenes, hence their name oncomirs. The first study involving miRNAs in cancer was in chronic lymphocytic leukemia (CLL) that showed miR-15 and miR-16 functioning as tumour suppressor genes (Calin, Ferracin et al. 2005). These observations might be of interest both for diagnostic and prognostic purposes.

Several studies have implicated TP53 in the regulation of miRNA expression (Raver-Shapira and Oren 2007; Tarasov, Jung et al. 2007; Kumamoto, Spillare et al. 2008). All studies identified members of the miR-34 family as direct transactivation targets of TP53. The precise function of *miR-34a* as a TP53 helper is unclear at this point, as some reports implicate miR-34a in cell cycle arrest whereas others suggest that *miR-34a* promotes apoptosis (Hermeking 2007).

## *5.2 Aim*

The hypothesis is that miR-34a is a potential target of TP53 that can act in synchrony with or independent of p21. The aim of this chapter was to determine the expression level

of miR-34a in DLBCL sections and to correlate that level with other immunohistochemical markers and the mutational status of *TP53* gene.

## *5.3 Materials and methods*

The expression of the miR-34a was investigated in sixty two cases belonging to DLBCL and Hep-G2 cell line using RT-PCR. Multiplex stem-loop RT-PCR with 18s was used as an endogenous RNA control. Normal tonsil was used as reference tissue. Details of RNA extraction and RT-PCR are given in the methods section.

## *5.4 Results*

### 5.4.1 Data analysis

$\Delta\Delta C_T$ studies were performed on the miRNA data using the SDS 2.1 software (Applied Biosystems, CA, USA). The studies were then exported to the Microsoft® Office Excel® 2007 software (Microsoft Corporation, WA, USA) for further analysis. There were two methods for ΔΔCt analysis. ΔΔCt values for each case was calculated in relation to normal tonsil as the reference tissue. For statistical reasons, MiR-34a expression was grouped into 3 groups of high, low and normal expression according to ΔΔCt interquatile range. ΔΔCt> 75%was defined as low expression, <25% as high expression and between 25-75% as normal expression.

### 5.4.2 Expression of miRNAs in DLBCL cases

The expression of the miR-34a was investigated in sixty two cases of classic DLBCL. Seven cases belonging to T-cell rich group as well as 3 cases (patient 8, 10 and 67), that failed to show expression by RT-PCR, were excluded from this analysis. The expression of miRNA-34a from 62 cases, normal tonsil and Hep-G2, a cell line with WT-p53 was assessed. Ct values for miR-34a were normalised using 18s rRNA using the equation ΔCt = $Ct_{(miR\text{-}34a)} - Ct_{(18s)}$ as shown in figure .High Δ CT (e.g. cases 52) corresponded to low

expression, while low ΔCT (e.g. cases 71) corresponded to high expression. All cases and HepG2 Δ Ct were correlated to ΔCT of normal tonsil as it represents the closest cell population and microenvironment of DLBCL. ΔΔCT were calculated from the equation $\Delta\Delta Ct = \Delta Ct_{(case)} - \Delta Ct_{(tonsil)}$.

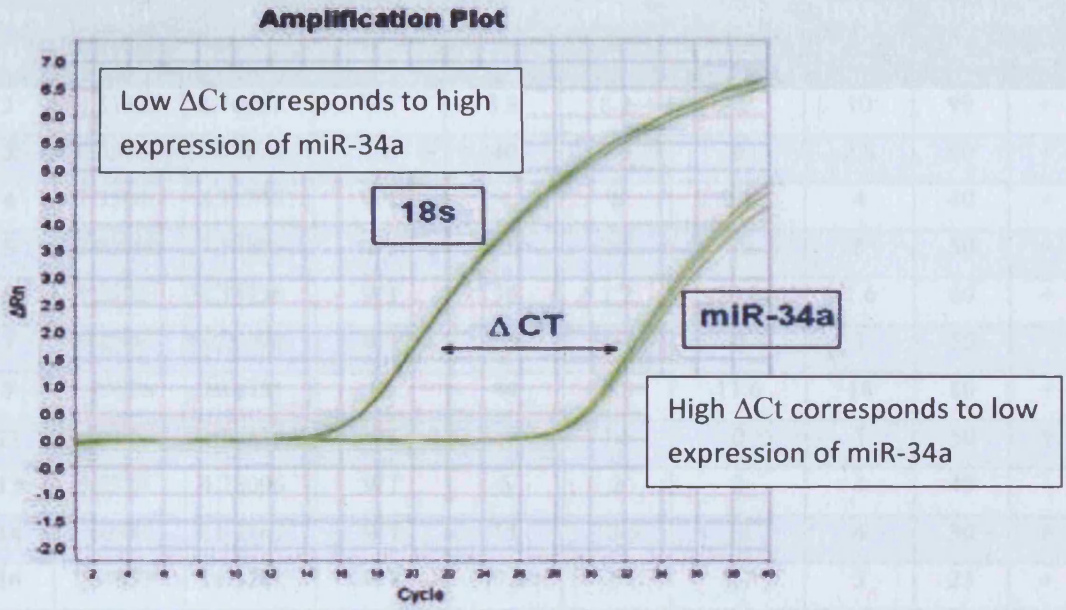



**Figure 5-1: An example amplification plot produced by qRT-PCR.** The figure shows fluorescence curves for miR-34a and 18s internal control in triplicate. The figure also illustrates how the ΔCt determines expression levels for the miRNAs; the Ct values are calculated by the qPCR machine using default threshold settings.

Fifteen cases (24.18%) had ΔΔCt values of miR-34a ranging from –2.03863 to –9.26184, corresponding to increased miR-34a expression compared to normal tonsil, thirty two (51.61%)cases had a ΔΔCt value ranging from –2.03863 to 2.0976, corresponding to normal miR-34a expression. Low expression of miR-34a was found in 15 cases (24.18%), with ΔΔCt ranging from 2.0976 to 5.40 as shown in Figure 5-1 and Table 5-1.

MiR-34a expression was grouped into 3 groups of high, low and normal expression according to ΔΔCt. ΔΔCt> 75%was defined as low expression, <25% as high expression and between 25-75% as normal expression. Twenty patients (32.2%) had a high expression of MiR-34a, 21 cases (33.9%) had nearly normal expression and 21 cases

(33.9%) had low expression. There was no correlation between miR-34a expression and age or gender.

**Table 5-1: Collective data of miR-34a, *TP53* mutational status, and TP53 and related proteins expression.**

| Cases ID | ΔΔCt | ΔCt | *TP53* gene | TP53% | p21% | Phospho-TP53 % | HDM2 % | MIB1 % | Bcl-2 | Bcl-6 |
|---|---|---|---|---|---|---|---|---|---|---|
| 2 | -3.31323 | 6.36063 | WT | 8.8 | 8.2 | 7.2 | 10 | 99 | + | - |
| 3 | -0.0571 | 9.61676 | M | 40 | 1.2 | 0 | 2.8 | 90 | + | - |
| 4 | -1.3564 | 8.31746 | WT | 4 | 6 | 2.8 | 4 | 40 | + | + |
| 5 | -2.62302 | 7.05085 | WT | 3.5 | 2.5 | 0 | 6 | 50 | - | - |
| 6 | -1.3365 | 8.33736 | WT | 36 | 1.2 | 20.8 | 1.6 | 60 | + | + |
| 7 | 1.02556 | 10.6994 | WT | 2.5 | 4 | 0 | 1 | 75 | + | + |
| 9 | 1.20126 | 10.8751 | M | 44 | 0.8 | 11.6 | 18 | 80 | + | - |
| 11 | 1.0081 | 10.6819 | WT | 9 | 1.5 | 0 | 3 | 50 | + | - |
| 13 | -5.3929 | 4.28096 | WT | 6 | 2 | 2 | 4 | 40 | - | - |
| 14 | 1.60286 | 11.2767 | WT | 15 | 13.5 | 12 | 4 | 30 | + | - |
| 16 | 1.64633 | 11.3202 | WT | 17.2 | 5 | 7.7 | 5 | 25 | + | - |
| 17 | 2.94363 | 12.6175 | M | 55.6 | 0.6 | 72.7 | 1.1 | 80 | + | - |
| 18 | -1.11377 | 8.5601 | WT | 21.1 | 0.6 | 61.1 | 1.1 | 70 | + | + |
| 19 | -1.7413 | 7.93256 | WT | 5 | 6 | 15 | 1 | 50 | - | + |
| 20 | 9.8805 | 9.8805 | WT | 4 | 2.5 | 2 | 0.5 | 50 | - | - |
| 21 | 3.3872 | 13.0610 | WT | 5.8 | 1.7 | 0 | 0.8 | 25 | + | - |
| 22 | 1.88506 | 11.5589 | WT | 9.4 | 20.6 | 1.8 | 0.5 | 50 | + | + |
| 23 | 0.0977 | 9.77156 | WT | 25 | 3.3 | 3.3 | 0.5 | 80 | + | - |
| 24 | 2.09018 | 11.7640 | WT | 10.6 | 10 | 4.4 | 0.5 | 50 | + | - |
| 25 | 10.8645 | 10.8645 | M | 45 | 1.5 | 85 | 1 | 70 | + | + |
| 26 | 2.41433 | 12.0882 | WT | 20.5 | 2.7 | 0 | 1.1 | 40 | - | - |
| 27 | 11.8634 | 11.8634 | WT | 5 | 1 | 2.5 | 0.5 | 50 | + | - |
| 28 | 3.90846 | 13.5823 | WT | 2.2 | 3 | 5 | 1 | 50 | + | - |
| 29 | 0.89263 | 10.5665 | WT | 21.5 | 3.5 | 6.5 | 2 | 50 | - | - |
| 30 | 4.85003 | 14.5239 | WT | 5 | 0.5 | 3.5 | 1 | 80 | + | + |
| 31 | 4.18316 | 13.8570 | WT | 27.5 | 0.5 | 2 | 11 | 90 | + | + |
| 32 | 4.07093 | 13.7448 | WT | 20.5 | 8.5 | 9 | 11.4 | 80 | - | + |
| 34 | 1.47913 | 11.153 | WT | 21.7 | 2.8 | 38.9 | 1 | 80 | - | - |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **35** | 11.3812 | 11.3812 | M | 46 | 1 | 35 | 3.3 | 75 | + | - |
| **36** | 9.42006 | 9.42006 | WT | 10.8 | 7.5 | 0 | 2.8 | 30 | - | + |
| **38** | 9.21436 | 9.21436 | M | 66.7 | 2 | 65.3 | 33.3 | 75 | - | - |
| **39** | 12.0190 | 12.0190 | WT | 4.7 | 5.3 | 7.1 | 26.6 | 50 | - | + |
| **40** | 11.1636 | 11.1636 | WT | 3.3 | 20 | 0 | 4 | 50 | + | + |
| **41** | 7.84190 | 7.84190 | WT | 10.5 | 5.5 | 8 | 19 | 60 | + | - |
| **42** | 7.32346 | 7.32346 | WT | 10 | 7.5 | 18 | 33.3 | 85 | + | + |
| **43** | 9.55333 | 9.55333 | WT | 9.3 | 1.3 | 16 | 20 | 70 | + | - |
| **45** | -0.83033 | 8.84353 | WT | 52.7 | 10 | 56.7 | 10.5 | 75 | - | - |
| **46** | -5.32354 | 4.35033 | WT | 12.5 | 1.5 | 4.5 | 28 | 80 | + | + |
| **48** | -4.9218 | 4.75206 | WT | 28.8 | 2.4 | 40 | 3.5 | 50 | - | - |
| **49** | -2.49416 | 7.17970 | WT | 16.4 | 0.71 | 3.5 | 18 | 75 | + | - |
| **50** | -6.81107 | 2.8628 | WT | 19 | 40 | 17.5 | 4 | 95 | + | - |
| **51** | 0.1791 | 9.85296 | WT | 5 | 1.5 | 3 | 3 | 75 | + | + |
| **52** | -0.23764 | 9.43623 | M | 30 | 2 | 90 | 13.3 | 75 | - | - |
| **53** | 5.39623 | 15.0701 | WT | 21.3 | 3.3 | 8 | 2.5 | 90 | + | + |
| **54** | 1.15470 | 10.8285 | M | 25 | 0.5 | 21 | 1 | 90 | + | + |
| **55** | -1.7087 | 7.96517 | WT | 20.5 | 0.5 | 70 | 13.8 | 80 | + | + |
| **56** | -0.23382 | 9.44005 | WT | 20 | 3.3 | 4.4 | 30 | 20 | + | + |
| **57** | 2.01559 | 11.6894 | WT | 21.1 | 1.11 | 3.3 | 11 | 50 | + | + |
| **58** | 2.1197 | 11.7935 | WT | 30 | 1.5 | 25 | 22 | 70 | + | + |
| **59** | 0.77030 | 10.4441 | WT | 10 | 0.5 | 4 | 2 | 80 | - | + |
| **60** | 1.66246 | 11.3363 | FS | 3 | 1 | 75 | 4 | 50 | + | + |
| **61** | -6.61473 | 3.05913 | WT | 3 | 11 | 8 | 5.5 | 60 | + | + |
| **62** | -4.13443 | 5.53943 | WT | 12.2 | 0.6 | 11.1 | 4 | 80 | + | + |
| **63** | -3.01104 | 6.66283 | WT | 7 | 0.5 | 2 | 5 | 90 | - | + |
| **64** | -4.92549 | 4.74837 | WT | 2 | 0.5 | 1 | 14.6 | 90 | - | + |
| **65** | -5.88103 | 3.79283 | S | 23.3 | 7.3 | 20 | 1.3 | 90 | - | - |
| **66** | -3.60653 | 6.06733 | WT | 20 | 0.7 | 36.7 | 2.5 | 80 | + | - |
| **68** | -5.87396 | 3.79990 | WT | 23.3 | 2.5 | 25 | 10 | 90 | - | + |
| **70** | 2.88813 | 12.562 | WT | 23 | 1 | 25 | 1.5 | 65 | - | + |
| **71** | -9.26184 | 0.41203 | WT | 4 | 3 | 2.5 | 10 | 75 | - | + |
| **72** | -1.5857 | 8.08816 | WT | 35 | 0.5 | 46 | 4 | 70 | + | - |
| **73** | -3.36007 | 6.31379 | M | 48 | 0.5 | 55 | | 90 | + | - |

*Cases 1, 15, 33, 37, 44, 47 and 69 were excluded from the study as they represented TCR subgroup. Cases 8, 10 and 67 failed to show RT-PCR amplification. WT: WT-p53, M: missense mutation, S: splice mutation, FS: Frame-shift mutation.*

### 5.4.3 Correlation between TP53 and miR-34a:

Normal expression of miR-34a was found equally distributed between TP53 positive and negative cases analyzed by IHC. A significant correlation was found between TP53 protein and miR-34a expressions ($p<0.05$), using Pearson correlation. Most of cases (10/15) with lower levels miR-34a had positive TP53 immunostaining, compared to normal tonsil. While 11 of 15 cases with high miR-34a expression were TP53 negative by immunohistochemistry as shown in table 5-2 and Figure 5-2. This possibly relates to presence of mutations in *TP53* gene that stabilizes the protein while abolishing his transcriptional activation of mi-R34a promoter.

The relationship between miR-34a and TP53 gene mutations was further analysed, Fourteen out of fifteen cases that showed high miR-34a expression levels, had a WT-p53 and the other case had a splice variant mutation in exon 9-intron 10 boundary. Normal miR-34a expression was found in 31 cases, five of them had a missense mutation in exon 5-8. While cases 10 cases with WT-p53 and 5 cases with TP53 mutations had low expression of miR-34a .Although there was a trend towards the association between high expression of miR-34a and WT-p53, that did not reach statistical significance ($p=0.57$). Yet with subsequent analysis of the mutation type, whether missense or other types, it was shown that the 9 cases with missense mutations identified, all had either low or normal miR-34a expression. No cases with missense mutations had a high miR-34a levels ($p=0.01$). Figure 5-3 shows the relationship between TP53 immunohistochemical scoring and $\Delta\Delta$Ct of miR-34a in 62 DLBCL cases in relation to *TP53* gene status.

Interestingly, mutations in codon 248 and 273which are considered mutational hotspots in haematological cancers, showed low expression of miR-34a Mutations at these particular sites are classified as contact (class I) mutants, which occur in DNA contact areas on either the L3 loop or the nearby loop-sheet-helix motif of *TP53*. The other

missense mutations found in this series are in codons 156, 184, 202, 255 and 282. All those mutants were identified in cases showing normal expression of miR-34a.

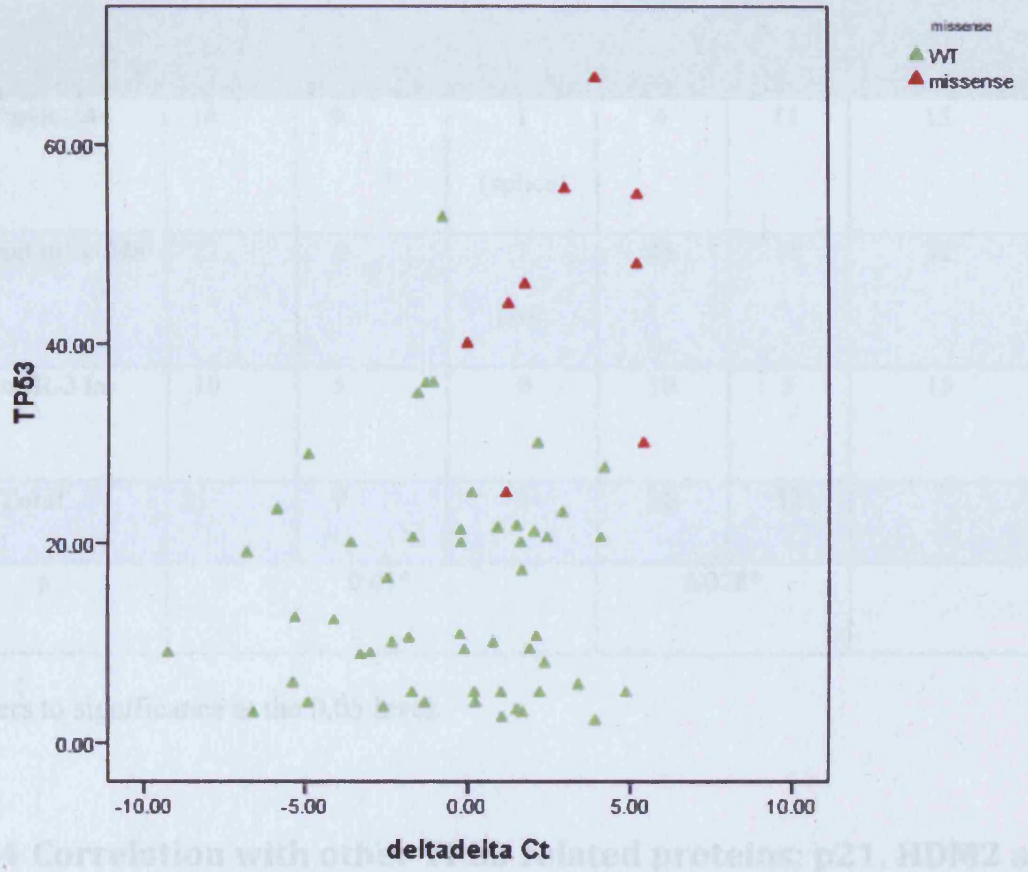



**Figure 5-2: The relationship between TP53 and ΔΔ Ct of miR-34a in 62 DLBCL cases**. High miR-34a levels correlated with low TP53 expression and WT-p53 and low miR-34a correlated with TP53 over-expression and missense mutations.

**Table 5-2 : Summary of miR34a, TP53 protein and mutational status**

| | *WT* | *Mutations* | | *TP53 protein* | | *Total* |
|---|---|---|---|---|---|---|
| | | *missense* | *other* | + | - | |
| **High miR-34a** | 14 | 0 | 1 (splice) | 4 | 11 | 15 |
| **Normal miR-34a** | 27 | 4 | 1 (FS) | 16 | 16 | 32 |
| **Low miR-34a** | 10 | 5 | 0 | 10 | 5 | 15 |
| **Total** | 51 | 9 | 2 | 30 | 32 | |
| p | 0.01* | | | 0.028* | | |

* refers to significance at the 0.05 level.

### 5.4.4 Correlation with other TP53 related proteins; p21, HDM2 and Phospho-p53:

Significant correlation was found between MiR-34a and p21 protein expression ($p<0.01$). Among 15 patients showing high expression of miR34-a, positive p21 staining was found in 9 cases (45%). While, 15 out of 15 cases with low expression of miR-34a, had a negative p21 immunostaining (100%). HDM2 showed a similar significant correlation with miR34a ($p<0.01$). Figure 5-3 shows the relationship between miR-34a, assessed by RT-PCR, and p21 and HDM2 protein expression assessed by immunohistochemistry. Figure 5-4 shows the correlation between miR-34a and p21 in relation to TP53 mutational status. phospho-specific p53, however, showed no correlation with miR-34a. ($p=0.2$) as shown in Table 5-3 and Figure 5-3.

**Table 5-3: The relationship between miR-34a expression and TP53, p21, HDM2 and Phospho-p53.**

| | TP53 | | p21 | | HDM2 | | Phosho-p53 | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | + | - | + | - | + | - | + | - | |
| **High miR-34a** | 4 | 11 | 9 | 6 | 6 | 9 | 4 | 11 | 15 |
| **Normal miR-34a** | 16 | 16 | 2 | 30 | 4 | 28 | 9 | 23 | 32 |
| **Low miR-34a** | 10 | 5 | 0 | 15 | 2 | 13 | 7 | 8 | 15 |
| **Total** | 30 | 32 | 11 | 52 | 12 | 50 | 20 | 42 | 62 |
| **p** | 0.028* | | 0.01* | | 0.01* | | 0.248 | | |

* refers to significance at the 0.05 level.

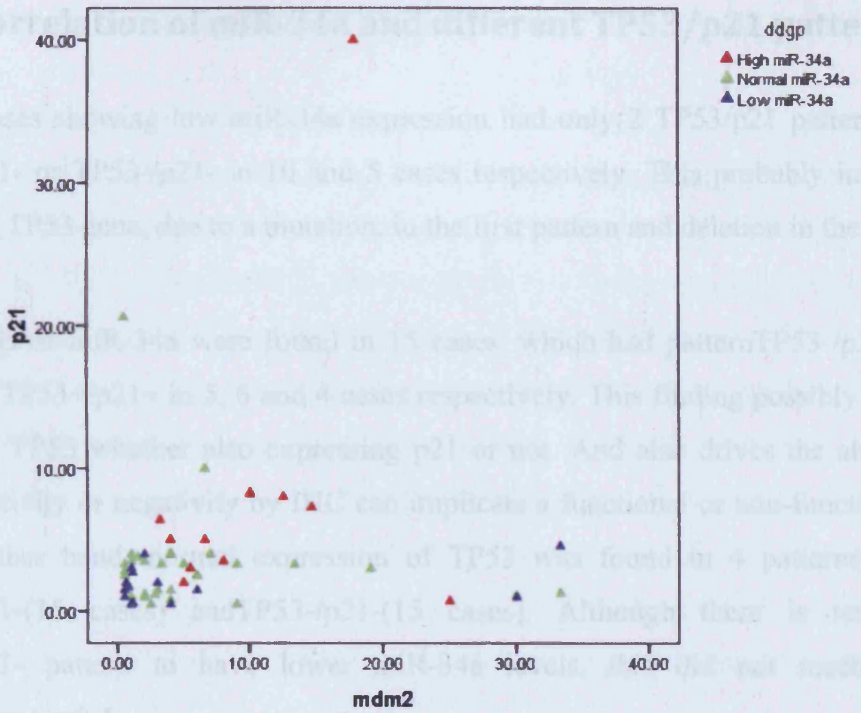



**Figure 5-3: The correlation between p21and HDM2 and miR-34a.** High miR-34a correlated with p21 and HDM2 over-expression. Low miR-34a correlated with low p21 and HDM2 expressions.

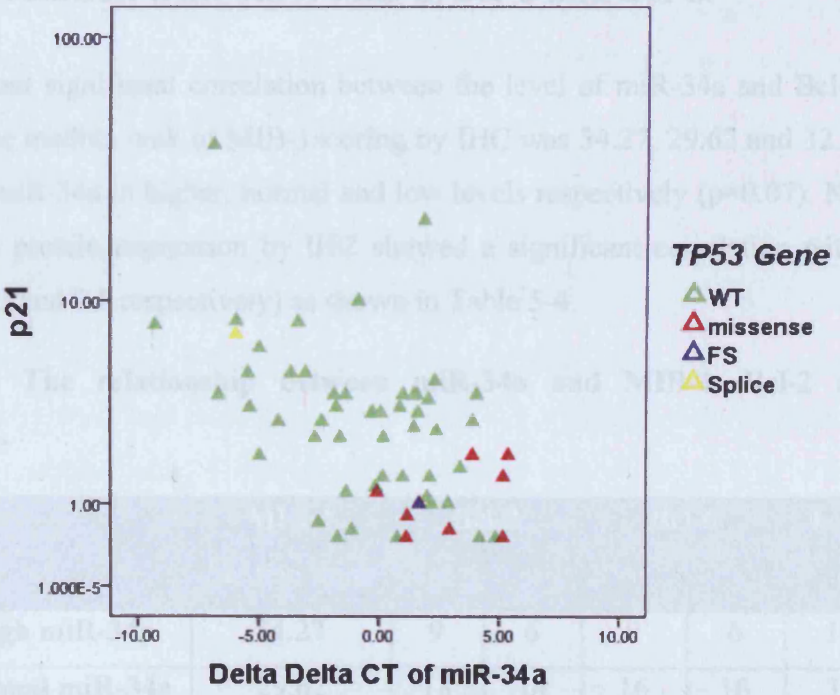



**Figure 5-2: The relationship of TP53 mutations and p21 and ΔΔCt of miR-34a**. All ten missense mutations and one FS mutations had a lower expression of p21 and miR-34a. WT p53 and a splice mutation in intron -10 had higher levels of p21 and miR-34a.

### 5.4.5 Correlation of miR-34a and different TP53/p21 patterns:

The 15 cases showing low miR-34a expression had only 2 TP53/p21 patterns namely; TP53+/p21- or TP53-/p21- in 10 and 5 cases respectively. This probably infers a non-functional TP53 gene, due to a mutation, in the first pattern and deletion in the second.

High levels of miR-34a were found in 15 cases, which had patternTP53-/p21+ ,TP53-/p21- and TP53+/p21+ in 5, 6 and 4 cases respectively. This finding possibly underlies a functional TP53 whether also expressing p21 or not. And also drives the attention that TP53 positivity or negativity by IHC can implicate a functional or non-functional TP53. On the other hand, normal expression of TP53 was found in 4 patterns mainly in TP53+/p21-(15 cases) andTP53-/p21-(15 cases). Although there is tendency for TP53+/p21- pattern to have lower miR-34a levels, this did not reach statistical significance (p=0.4).

### 5.4.6 Correlation with other MIB-1, Bcl-2 and Bcl-6:

There was not significant correlation between the level of miR-34a and Bcl-2 or Bcl-6 proteins. The median rank of MIB-1scoring by IHC was 34.27, 29.62 and 32.73 in cases expressing miR-34a in higher, normal and low levels respectively (p=0.07). Neither Bcl-2 nor Bcl-6 protein expression by IHC showed a significant correlation with miR-34a levels (p=0.3 and 0.5 respectively) as shown in Table 5-4.

**Table 5-4: The relationship between miR-34a and MIB-1, Bcl-2 and Bcl-6 expressions.**

| | MIB-1 | Bcl-2 | | Bcl-6 | | Total |
|---|---|---|---|---|---|---|
| | Median Rank | + | - | + | - | |
| **High miR-34a** | 34.27 | 9 | 6 | 9 | 6 | 15 |
| **Normal miR-34a** | 29.62 | 18 | 14 | 16 | 16 | 32 |
| **Low miR-34a** | 32.73 | 5 | 10 | 10 | 5 | 15 |
| p | 0.67 | 0.3 | | 0.5 | | |

### 5.4.7 Summary of results

miR-34a expression levels were investigated in 62 cases of DLBCL. Expression levels was classified as high, normal and low in 24.18%, 51.6% and 24.18% respectively. A significant correlation was found between TP53 protein and miR-34a expressions ($p<0.05$). Yet, the striking finding in this study, is the strong association of the presence of TP53 mutations and low level of miR-34a ($p<0.01$). Mutations in codons 248 and 273, both classified as Class I mutant, were the most frequent among cases showing low level of miR-34a. No correlation was found between miR-34a and phospho-TP53, MIB-1, Bcl-2 or Bcl-6 yet it showed a strong correlation with p21 and HDM2 ($p <0.05$). In this series, there was a strong correlation between the expression of both p21 and miR-34a. Whether this a causal issue relating to TP53 being WT in some cases and thus inducing miR-34 which regulates p21 or just inducing both of them independently is not clear at this point.

These data suggest that WT p53 found in this cohort of cases was able to induce several downstream effectors. One of them utilizes an accurate and energy efficient way of controlling the expression of several proteins; this highlights the potential role of MiR-34a as a potential TP53 target.

## *5.5 Discussion*

With the recent discovery of noncoding RNAs, (miRNA) and small interfering RNAs (siRNA), as translational regulators, it is clear that miRNAs play important roles in regulating gene expression. Translational control has been shown to play a fundamental role in oncogenesis (Sheikh and Fornace 1999).

miR-34 family contains three members; miR-34a , b and c, coded by two loci in the vertebrate genome, one on chromosome 1p36 encoding miR-34a while both miR-34b and miR-34c share a common primary transcript from chromosome 11q23. miR-34a is expressed at higher levels than miR-34b/c in all tissues, with the exception of the lung,

where miR-34b/c is dominantly expressed (Bommer, Gerin et al. 2007). Several studies using chromatin immunoprecipitation (ChIP) and luciferase reporters assay, showed a presumptive TP53 binding region within 30 kb of the precursor transcription units for both miRNA34a and miRNA34b/c. TP53's functions as a transcription factor are well documented, and thus miRNAs acting via RNA interference pathway (RNAi), offered the TP53 the possibility to indirectly repress target gene expression at the posttranscriptional level.

miR-34a expression levels was investigated in 62 of DLBCL, miR-34a correlated with TP53 protein expression ($p<0.05$), but not with phospho-p53. Interestingly, the presence of missense mutations in *TP53* associated with a lower level of miR-34a expression ($p=0.01$). All cases with a missense *TP53* mutation identified by SSCP and confirmed by sequencing had either low or normal expression of miR-34a, but none had a high level of expression. Codon position 248 was the most frequent target of mutation in all series and especially among cases with low expression of miR-34a (3/5 cases). This mutational hotspot site resides where DNA contact occur and hence classified as contact mutant (Cho, Gorina et al. 1994). The fourth mutation was in codon 273, also a contact (class I) mutant. While the fifth mutation was in codon 282, known as denaturing or conformational (class II) mutant, affecting the stability of TP53. Obviously this is a simplified view of those mutations as they can share some of each others' properties. As, some conformational mutants can retain some of DNA binding capabilities and vice versa.

This finding correlates to several studies on the role of *TP53* in the induction of miR-34 although they were done in vitro. Tarasov and colleages performed a genome-wide screening for microRNAs regulated by the transcription factor encoded by *TP53*. By conditional activation of TP53 in H1299 lung cancer cells, carrying a homozygous deletion of the *TP53* locus, and monitoring the levels of miRNA induced or inhibited by TP53 activation. Amid thirty-four miRNAs exhibiting significantly increased expression, the induction of miR-34a was most pronounced among all differential regulations (Tarasov, Jung et al. 2007). In 2008, Ji et al transfected TP53-deficient human gastric cancer cells with miR-34 mimics. Their results suggest that the restoration of miR34 can

inhibit tumour formation and growth via inhibition of Bcl2, a key inhibitor of apoptosis, Notch, and HMGA2 (Qing Ji 2008).

In DLBCL, there was a strong correlation between the expression of both p21 and miR-34a ($p < 0.01$). This could indicate that in some cases investigated in this study, functional TP53 is capable of inducing both p21 and miR-34a and thus acting on the transcriptional and posttranscriptional regulation of the cell cycle. This was reported in a study by Paris et al who showed that the cellular response to Nutlin-3, a small-molecule inhibitor of the TP53 repressor HDM2, varies widely among human cancer derived cell types. They reported that TP53 have several players at its disposal in the apoptotic machinery, namely p21, miR34a and *14-3-3*$\sigma$ (Paris, Henry et al. 2008).

The other explanation is that miR-34 itself can modulate the expression of p21. This was reported by Yamakuchi et al (2008), showing that miR34a can increase expression of p21 and PUMA, another transcriptional targets of TP53. Moreover they showed that miR-34a can repress silent information regulator 1(SIRT-1), a NAD-dependent deacetylase and increases acetylated TP53 thus activating the TP53 signalling pathway (Yamakuchi, Ferlito et al. 2008).

There is also other evidence that other miRNAs can regulate p21 and other cdk-inhibitors. For example, a recent study showed that miR-106 and miR-93, which are up regulated in stomach cancer cells, can down regulate p21 (Ivanovska, Ball et al. 2008; Kim, Yu et al. 2009). Another study found that miR-192 and miR-215 can act as targets as well as regulators of TP53; that seem to suppress cancerogenesis through p21 accumulation and cell cycle arrest (Braun, Zhang et al. 2008).

There was a significant correlation between miR-34a and HDM2 ($p < 0.01$). In this series, HDM2 was shown to act more as a TP53 target as shown from HDM2 and miR-34a expression levels. The relationship between TP53 and HDM2 is very complex. First HDM2 is induced by TP53. Secondly HDM2 acting as an ubiquitin ligase will induce TP53 degradation. Concomitant Lack of HDM2 and miR-34a expression is probably the result of non-functional TP53. This was reported in the study by Paris et al, giving

evidence that after TP53 activation of p21, miR34a, PUMA and PIGs (p53- inducible genes), similar increase in HDM2 was observed (Paris, Henry et al. 2008).

Recently, the ability of HDM2 to induce TP53 has been discovered. HDM2 stimulates TP53mRNA translation by binding the TP53 mRNA which meanwhile also suppresses HDM2's capacity to promote TP53 polyubiquitination and degradation. So the effect of HDM2 on TP53 expression is actually a balance between synthesis and degradation (Candeias, Malbert-Colas et al. 2008).

# Chapter 6

# Discussion

# 6 Discussion, conclusions and future work

## *6.1 Discussion*

Since the description of TP53 as the Guardian of the genome by David Lane, it has been the most extensively studied tumour suppressor protein (Lane and Crawford 1979). The TP53 gene product is a 393 amino acid nuclear phosphoprotein, which under normal circumstance, is a short lived protein that attains a latent conformation. Upon its activation by several types of genotoxic stresses like DNA damage, hypoxia, oncocogene activation and viral replication, TP53 have several pathways at its disposal (Levine 1997). Among these, are growth arrest, apoptosis, and cellular senescence.

The *TP53* gene is the most frequent target of genetic alterations, being mutated in more than half of human cancers (Iwakuma, Lozano et al. 2005). The mutant TP53 proteins first lack the WT-p53 activity and second can have new gain of function properties that promote oncogenesis (Kim and Deppert 2004). Most of *TP53* mutations are missense, which is quite unique among other tumour suppressor genes. Most tumour suppressor genes are inactivated by frame shift or nonsense mutations leading to the complete elimination of the gene products or the production of truncated proteins which are both non-functional (Blagosklonny 2000).

DLBCL is the most common aggressive NHL and comprises nearly 40% of adult lymphomas. The disease represents a clinically and genetically heterogeneous entity. The disease has a poor prognosis in half of the patients because of its aggressive and highly proliferating nature. With the introduction of Rituximab, the overall survival has been improved (Habermann, Weller et al. 2006). In DLBCL, TP53 is of heightened interest regulating the cell proliferation and apoptosis. The frequency of *TP53* mutations in haematological malignancies is less than solid tumours, with mean of 14%, but they carry unfavourable prognosis relative to WT-p53 (Peller and Rotter 2003).

In this study, several TP53 related proteins were assessed along with routinely used markers for DLBCL diagnosis. These include; p21 as the most important downstream target of TP53, HDM2 the best known regulator of TP53 and Phospho-specific TP53 as a marker of activation of TP53. These were analysed in context with the molecular data gathered from screening for *TP53* mutations in exons 5-9 by SSCP-sequencing and the recently discovered TP53 target namely miR-34a.

The study involved 72 cases of DLBCL retrieved from the UHL pathology archive over the period from 2000-2006. These included seven TCR cases and 65 classic DLBCL cases. TP53 over-expression ($\geq$ 20%) was observed in 52.8% of DLBCL cases. The frequency of TP53 over-expression in the literarture varies from 27% (Leroy, Haioun et al. 2002) to 56% (Paik, Jeon et al. 2005). This could be explained by differences in the cut-offs used, the fixation and the antigen retrieval methods used (Olapade-Olaopa, MacKay et al. 1998; van den Broek and van de Vijver 2000). It of special importance to note that TP53 over-expression can be detected in rapidly proliferating normal and neoplstic haematopoietic cells (Danova, Giordano et al. 1990).

Because molecular analysis of TP53 is not carried out routinely in most laboratories, several attempts have been made to use TP53 immunohistochemistry as a surrogate marker for TP53 mutations (Maestro, Gloghini et al. 1997). In this series there was no significant correlation between TP53 positivity and the presence of mutation ($p=0.33$) and thus this hypothesis cannot be proven based on this study.

However the findings of this series, where no mutation was detected in 20 out of 31 cases with high TP53 levels ($p<0.005$), do suggest that the nuclear or cytoplasmic stabilization of TP53 protein could also depend on other factors, as has been described in other tumours such as soft-tissue sarcomas (Oliner, Pietenpol et al. 1993). Similarly the TP53 phosphorylated at Serine 392 showed a significant correlation of its negativity by immunohistochemistry (40/43 cases, $p\leq0.005$) and the absence of mutation of *TP53* gene. Nevertheless, there was no correlation between its positivity and the presence of such mutation (9 of 21 cases, $p=0.591$). Similar findings were reported in several

studies showing that TP53 expression is independent of *TP53* mutations (Oka, Sarker et al. 1998; Sanchez-Beato, Sanchez-Aguilera et al. 2003).

This has led to the establishment of four TP53/p21 expression patterns to further assess the TP53 functionality. This is based on the ability of TP53 to cause cell cycle arrest via its main downstream target, p21. A TP53+/p21- phenotype was found in 28 of 72 cases and was correlated to *TP53* missense mutations ($p<0.005$). This in concordance to several reports suggesting the role of *TP53* missense mutations in the production of a more stable protein (TP53+), while abolishing its ability to induce p21 (p21-) (Villuendas, Piris et al. 1992; Visco, Canal et al. 2006).

In this series, there was a strong correlation between TP53 and MIB-1 ($p<0.005$) and phospho-p53 ($p<0.005$). There was also a strong negative correlation between and TP53 and Bcl-2 expressions ($p<0.005$). In this study, all phospho-p53 positive tumours were positive for TP53 protein expression detected by DO-1. Yap et al 2004 have shown that phosphorylation of TP53 at serine 392 phosphorylation had a clear effect on the anti-apoptotic function of mutant TP53, which was independent of the DNA binding activity of TP53. In this study, phospho-p53 positive tumours showed a high proliferation index compared to phospho-p53 negative tumours. A similar finding was also reported by Matsumoto et al (2004) where Serine 392 phosphorylation of TP53 was significantly associated with proliferative activity of tumour cells (Matsumoto, Furihata et al. 2004).

It was also observed that rapidly proliferating tumours show over-expression of TP53 ($p<0.005$) and was correlated to TP53 mutations ($p<0.005$) which has been reported before in lymphomas (Cesarman, Inghirami et al. 1993). There was a negative correlation between the proliferation index and the anti-apoptotic protein Bcl-2 ($p<0.005$). A similar finding was shown by other groups (Bai, Agnantis et al. 2003; Jerkeman, Anderson et al. 2004), keeping the notion that anti-apoptotic protein , Bcl-2, can inhibit cellular proliferation (Janumyan, Sansam et al. 2003). While a clear relationship was not observed in a recent study examining paediatric DLBCL (Miles, Raphael et al. 2008).

This relationship observed in this study, between TP53 and Bcl-2, is similar to other groups who showed that WT-p53 down-regulate the anti-apoptotic Bcl-2 protein and the loss of this repression may lead to up-regulation of Bcl-2 by the previous identification of a TP53-dependent negative response element in the bcl-2 gene (Miyashita, Harigai et al. 1994). Similar finding was also reported in other types of lymphomas such as FL, gastric and primary bone lymphomas (Huebner-Chan, Fernandes et al. 2001).

The frequency of *TP53* mutations was 18.75% in this study, which is comparable to the average frequency of several reports (Leroy, Haioun et al. 2002; Young, Weisenburger et al. 2007). All of the mutations identified were previously reported. Exon 7 harboured most of the mutations (30%), especially at codon 248 which is considered a mutation hot-spot. The distribution of *TP53* mutations among different exons is variable in the literature, mostly reporting exon 7 as the most common target of inactivating mutations. Other exons constitute less frequent targets of such mutation, with exon 9 representing the least of them. This relates to the frequency reported by IARC and p53 knowledge database (Hainaut, Hernandez et al. 1998).

The HDM2 gene is a cellular proto-oncogene that is often amplified in less than 10 % of all human cancers, but most frequently in soft tissue sarcomas (Momand, Jung et al. 1998). HDM2, as an ubiquitin ligase, regulates the activity of TP53 protein by targeting TP53 for proteasomal degradation or enhance TP53 nuclear export where it is degraded in the cytoplasm. HDM2 protein overexpression ($\geq$10%) was found in 23.6% of cases. Strong positive correlation was found between p21 and HDM2 protein expression ($r=0.3337$, $p<0.005$). Yet no correaltion was found between individual HDM2 or p21 and TP53 overexpression and mutations which is similar to other reports (Villuendas, Piris et al. 1992).

Recently, a small class of RNAs (miRNAs) was found to act as TP53 helpers after their known roles in cancer has been established (le Sage and Agami 2006). The miR-34 family, particularly miR-34a, has been catching the spotlight as being direct TP53 targets (Bommer, Gerin et al. 2007) based on the discovery of a presumptive TP53

binding domain in the precursor transcription units of both miR-34a and miR-34b/c (Wei,Wu et al 2006). miR-34a expression levels was assessed in 62 DLBCL cases. miR-34a correlated with TP53 protein expression ($p<0.05$) and with *TP53* missense mutations ($p=0.01$). This finding correlates to several in-vitro studies (Bommer, Gerin et al. 2007) , but to my knowledge this is first study reporting the relationship between TP53 and miR-34a in DLBCL.

Another interesting finding that arise from this study, is the correlation between the expression of both p21 and HDM2 and miR-34a ($p <0.05$). Whether it is based on the ability of functional TP53 to concomittently induce theses proteins or that miR-34a can regulate the expression of p21 and HDM2 is not clear at this point. Both explanations could be valid based on previous studies supporting the two hypotheses (Paris, Henry et al. 2008 and Yamakuchi, Ferlito et al. 2008). A summary of the finding observed in this syudy, is shown in Figures 6-1 and 6-2.

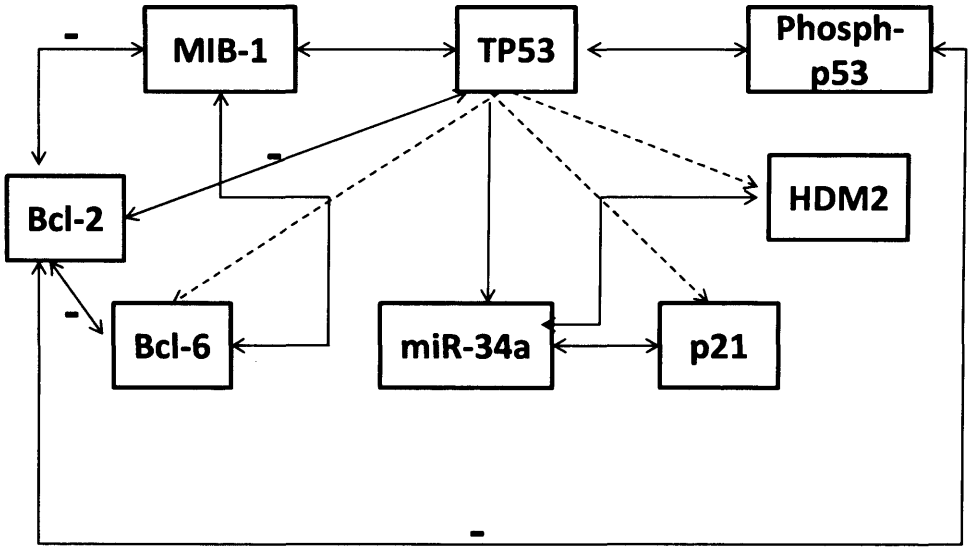



**Figure 6-1: TP53 in relation to immunhistochemical markers.** A strong positive correlation was found between TP53 and MIB-1 ($p<0.005$) and phopho-p53 ($p<0.005$). A strong negative correlation was found between TP53 and Bcl-2 ($p<0.005$) and miR-34a ($p<0.05$). No correlation was found between TP53 and p21, HDM2 or Bcl-6.

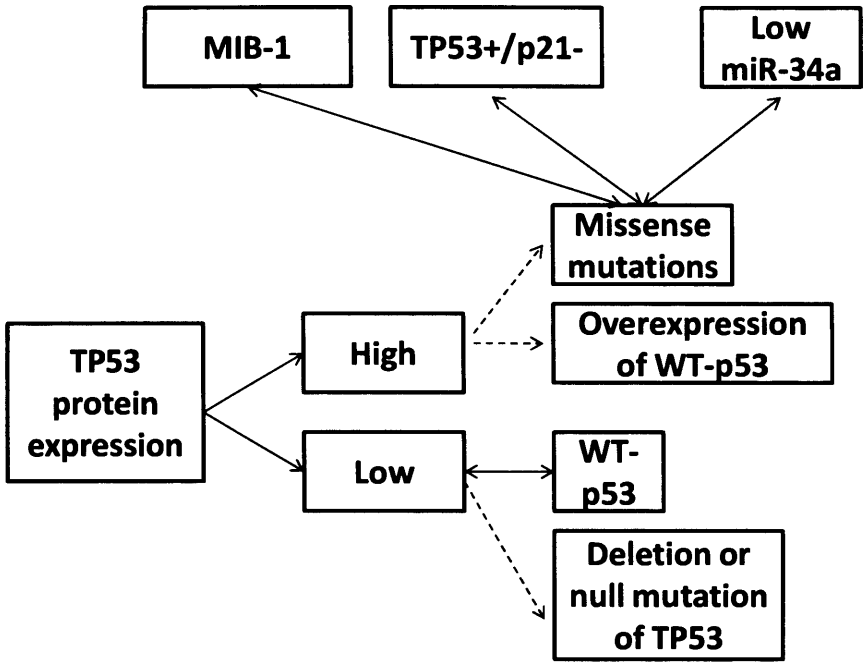



**Figure 6-2: *TP53* mutational status.** TP53 over-expression can be due to missense mutations causing a conformational change in TP53 that leads to its stabilisation, or due to over-activation of TP53 in response to carcinogenic stress. On the other hand low expression of TP53 can be due to the presence of WT-p53 or due to loss of *TP53* by deletions or null mutation. In this study, missense *TP53* mutations showed a correlation with low mi-34a ($p<0.05$), the presence of a TP53+/p21- phenotype ($p<0.005$) and high proliferation index ($p<0.005$).

## *6.2 Fulfilment of the aims:*

The aims of this thesis have been achieved, by molecular analysis of 64 cases of DLBCL and relating the findings to the expression of Tp53 and related proteins and miR-34a. In conclusion, this study showed a significant positive correaltion between TP53 and MIB-1, phospho-p53 and negative correlation between TP53 and Bcl-2 and miR-34a. *TP53* missnse mutations showed a correlation with low miR-34a, the presence of a TP53+/p21- phenotype and high proliferation index.

There was no correlation, however, between TP53 and its main downstream target (p21) or with its main regulator (HDM2). This suggests that not all cases with WT-p53 are fully functional and are not all phenotypically similar. Other mechanisms for

inactivating TP53 must be responsible, such as alterations of the downstream or upstream regulators or targets that will mimic a TP53 aberration.

## *6.3 Future Directions*

The delineation of DLBCL into GC and ABC subtype could provide further insights in relation to TP53 pathway. These will need further characterisation of cases by CD10 and MUM-1; which was not feasible in this study because of the lack of available sections. Other TP53 regulators, apart from HDM2 should also be addressed. These include COP-1, PIRH-2 and other HDM2 family members such as HDM-4 and HDM-X. Also the study of other TP53 isoforms as well as p63 and 73 in DLBCL will be helpful to capture the full picture of TP53 network in those lymphomas. Although it has a low frequency in lymphomas, other TP53 exons, outside the hotspot regions should also be screened for the presence of mutation.

It would be also useful to have survival data to assess the prognostic relevance of the markers studied. Unfortunately due to ethical restrictions, this was not done in this study. Acquiring a pre and post-transformation sections as well as pre and post relapses would be very helpful to show if TP53 or any of the markers could be used to predict the clinical behaviour of DLBCL.

To further unravel the connection between TP53 and the miR-34 genes, it will be important to define the targets of miR-34s in more detail using additional approaches, e.g., proteomic analyses. Furthermore, the relevance of the individual target downregulations mediated by miR-34s for tumor suppression by TP53 needs to be determined. Knock-out approaches combined with mouse models of cancer will presumably allow the function of miR-34 genes and their relevance for tumour suppression in vivo to be determined. In addition, it will be interesting to determine to what extent TP53-induced miRNAs or direct TP53-mediated repression account for the downregulation of genes observed after TP53 activation. Which pathway takes the upper hand or occurs first is still not understood.

Small interfering RNAs (siRNAs) are currently tested and optimized for clinical applications (de Fougerolles, Vornlocher et al. 2007). Therefore, it may be possible to restore miR-34 function for cancer therapeutic purposes in the future. Given the tumor-suppressive functions of the miR-34 family, it will be interesting to determine whether detection of miR-34a has diagnostic or prognostic advantages as previously shown for other miRNAs (Calin and Croce 2006)

Another relative of miRNA, piwi-interacting RNAs (piRNAs) were discovered in rat germline cells that play a role in gene regulation (Lau, Seto et al. 2006). Acting via RNA interference means that a rapid repression can be achieved without change of the steady state levels of mRNA. Thus the world of these small RNAs is providing new insights about genome control in normal and malignant cells. Finally, while miR-34a seems to catch the spotlight, other family members miR-34b/c also play a role in a tissue specific context. Other mi-RNAs induced by TP53 remains to undiscovered. A summary of the proposed other markers that can further unravel the TP53 pathway is given in Figure 6-3.

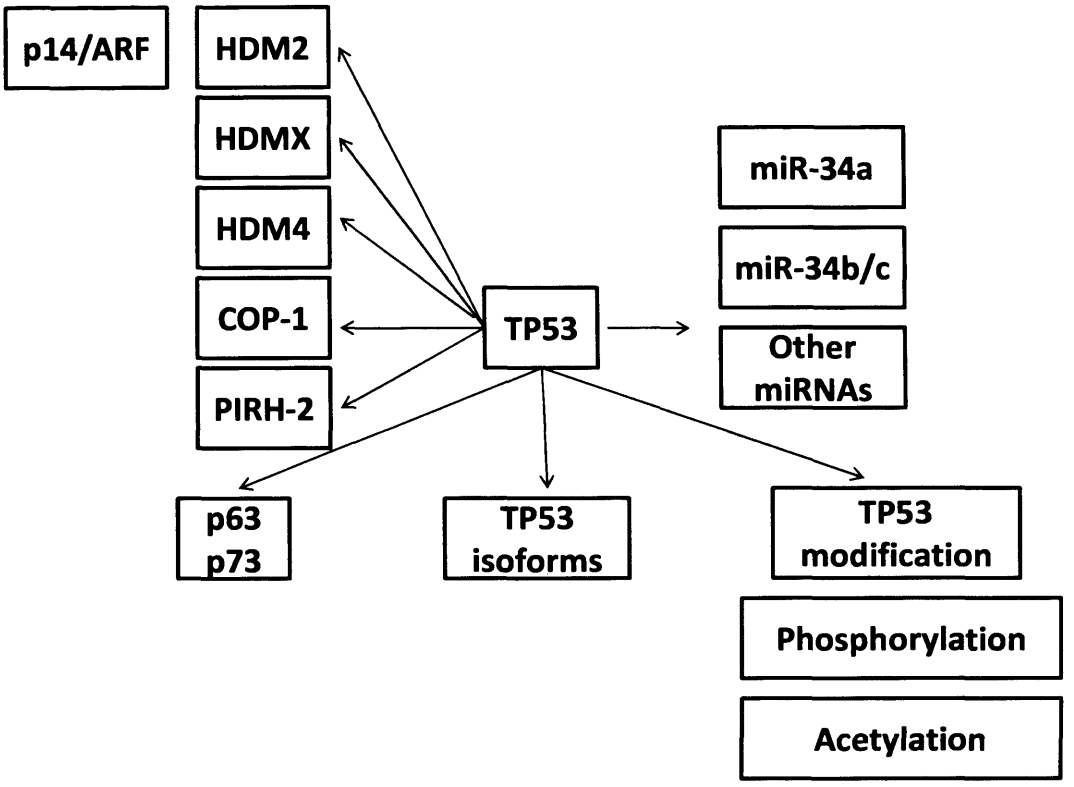



**Figure 6-3: Recommended markers for future studies.** These include other miRNAs; such as miR-15 and miR-16, other TP53 modifications; including other phosphorylation sites, TP53 isoforms, TP53 family members and other ubiquitin ligases and p14.

# Chapter 7

# References

Abdel-Fattah, G., B. Yoffe, et al. (2000). "MDM2/p53 protein expression in the development of colorectal adenocarcinoma." J Gastrointest Surg **4**(1): 109-14.

Adams, J. M. and S. Cory (1998). "The Bcl-2 protein family: arbiters of cell survival." Science **281**(5381): 1322-6.

Adamson, D. J., W. D. Thompson, et al. (1995). "p53 mutation and expression in lymphoma." Br J Cancer **72**(1): 150-4.

Agarwal, M. L., W. R. Taylor, et al. (1998). "The p53 network." J Biol Chem **273**(1): 1-4.

Alexander, D. D., P. J. Mink, et al. (2007). "The non-Hodgkin lymphomas: a review of the epidemiologic literature." Int J Cancer **120 Suppl 12**: 1-39.

Alizadeh, A. A., M. B. Eisen, et al. (2000). "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling." Nature **403**(6769): 503-11.

Amara, K., M. Trimeche, et al. (2008). "Presence of simian virus 40 in diffuse large B-cell lymphomas in Tunisia correlates with germinal center B-cell immunophenotype, t(14;18) translocation, and P53 accumulation." Mod Pathol **21**(3): 282-96.

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell **116**(2): 281-97.

Batta, K. and T. K. Kundu (2007). "Activation of p53 function by human transcriptional coactivator PC4: role of protein-protein interaction, DNA bending, and posttranslational modifications." Mol Cell Biol **27**(21): 7603-14.

Benard, J., S. Douc-Rasy, et al. (2003). "TP53 family members and human cancers." Hum Mutat **21**(3): 182-91.

Bendig, I., N. Mohr, et al. (2004). "Identification of novel TP53 mutations in familial and sporadic cancer cases of German and Swiss origin." Cancer Genet Cytogenet **154**(1): 22-6.

Berkson, R. G., J. J. Hollick, et al. (2005). "Pilot screening programme for small molecule activators of p53." Int J Cancer **115**(5): 701-10.

Bhatia, K., M. I. Gutierrez, et al. (1992). "PCR detection of a neutral CGA/CGG dimorphism in exon 6 of the human p53 gene." Nucleic Acids Res **20**(4): 928.

Bommer, G. T., I. Gerin, et al. (2007). "p53-mediated activation of miRNA34 candidate tumor-suppressor genes." Curr Biol **17**(15): 1298-307.

Bougeard, G., J. M. Limacher, et al. (2001). "Detection of 11 germline inactivating TP53 mutations and absence of TP63 and HCHK2 mutations in 17 French families with Li-Fraumeni or Li-Fraumeni-like syndrome." J Med Genet **38**(4): 253-7.

Bourdon, J. C., K. Fernandes, et al. (2005). "p53 isoforms can regulate p53 transcriptional activity." Genes Dev **19**(18): 2122-37.

Bouska, A. and C. M. Eischen (2009). "Mdm2 affects genome stability independent of p53." Cancer Res **69**(5): 1697-701.

Brauninger, A., T. Spieker, et al. (2003). "Epstein-Barr virus (EBV)-positive lymphoproliferations in post-transplant patients show immunoglobulin V gene mutation patterns suggesting interference of EBV with normal B cell differentiation processes." Eur J Immunol **33**(6): 1593-602.

Bruins, W., O. Bruning, et al. (2008). "The absence of Ser389 phosphorylation in p53 affects the basal gene expression level of many p53-dependent genes and alters the biphasic response to UV exposure in mouse embryonic fibroblasts." Mol Cell Biol **28**(6): 1974-87.

Bai, M., N. J. Agnantis, et al. (2003). "Increased expression of the bcl6 and CD10 proteins is associated with increased apoptosis and proliferation in diffuse large B-cell lymphomas." Mod Pathol **16**(5): 471-80.

Bertheau, P., D. Cazals-Hatem, et al. (1998). "Variability of immunohistochemical reactivity on stored paraffin slides." J Clin Pathol **51**(5): 370-4.

Blagosklonny, M. V. (2000). "p53 from complexity to simplicity: mutant p53 stabilization, gain-of-function, and dominant-negative effect." FASEB J **14**(13): 1901-7.

Bommer, G. T., I. Gerin, et al. (2007). "p53-mediated activation of miRNA34 candidate tumor-suppressor genes." Curr Biol **17**(15): 1298-307.

Braun, C. J., X. Zhang, et al. (2008). "p53-Responsive micrornas 192 and 215 are capable of inducing cell cycle arrest." Cancer Res **68**(24): 10094-104.

Burkitt, D. P. (1983). "The discovery of Burkitt's lymphoma." Cancer **51**(10): 1777-86.

Cadwell, C. and G. P. Zambetti (2001). "The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth." Gene **277**(1-2): 15-30.

Calin, G. A., M. Ferracin, et al. (2005). "A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia." N Engl J Med **353**(17): 1793-801.

Calin, G. A. and C. M. Croce (2006). "MicroRNA signatures in human cancers." Nat Rev Cancer **6**(11): 857-66.

Calin, G. A., C. D. Dumitru, et al. (2002). "Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia." Proc Natl Acad Sci U S A **99**(24): 15524-9.

Candeias, M. M., L. Malbert-Colas, et al. (2008). "p53 mRNA controls p53 activity by managing Mdm2 functions." Nat Cell Biol.

Caron de Fromentel, C. and T. Soussi (1992). "TP53 tumor suppressor gene: a model for investigating human mutagenesis." Genes Chromosomes Cancer **4**(1): 1-15.

Cattoretti, G., C. Angelin-Duclos, et al. (2005). "PRDM1/Blimp-1 is expressed in human B-lymphocytes committed to the plasma cell lineage." J Pathol **206**(1): 76-86.

Cayrol, C., M. Knibiehler, et al. (1998). "p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells." Oncogene **16**(3): 311-20.

Cevec, M., C. Thibaudeau, et al. (2008). "Solution structure of a let-7 miRNA:lin-41 mRNA complex from C. elegans." Nucleic Acids Res **36**(7): 2330-7.

Cesarman, E., G. Inghirami, et al. (1993). "High levels of p53 protein expression do not correlate with p53 gene mutations in anaplastic large cell lymphoma." Am J Pathol **143**(3): 845-56.

Chapusot, C., M. Assem, et al. (2001). "[Expression of p21 WAF1/CIP1 protein in colorectal cancers: study of its relation to p53 mutation and Ki67 antigen expression]." Pathol Biol (Paris) **49**(2): 115-23.

Cheah, P. L. and L. M. Looi (2001). "p53: an overview of over two decades of study." Malays J Pathol **23**(1): 9-16.

Chiorazzi, N. and M. Ferrarini (2003). "B cell chronic lymphocytic leukemia: lessons learned from studies of the B cell antigen receptor." Annu Rev Immunol **21**: 841-94.

Chilosi, M., C. Doglioni, et al. (1996). "p21/WAF1 cyclin-kinase inhibitor expression in non-Hodgkin's lymphomas: a potential marker of p53 tumor-suppressor gene function." Blood **88**(10): 4012-20.

Cho, Y., S. Gorina, et al. (1994). "Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations." Science **265**(5170): 346-55.

Cimmino, A., G. A. Calin, et al. (2005). "miR-15 and miR-16 induce apoptosis by targeting BCL2." Proc Natl Acad Sci U S A **102**(39): 13944-9.

Corcoran, C. A., Y. Huang, et al. (2004). "The p53 paddy wagon: COP1, Pirh2 and MDM2 are found resisting apoptosis and growth arrest." Cancer Biol Ther **3**(8): 721-5.

Corney, D. C., A. Flesken-Nikitin, et al. (2007). "MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth." Cancer Res **67**(18): 8433-8.

Cummins, J. M. and V. E. Velculescu (2006). "Implications of micro-RNA profiling for cancer diagnosis." Oncogene **25**(46): 6220-7.

Danova, M., M. Giordano, et al. (1990). "Expression of p53 protein during the cell cycle measured by flow cytometry in human leukemia." Leuk Res **14**(5): 417-22.

Dansonka-Mieszkowska, A., A. H. Ludwig, et al. (2006). "Geographical variations in TP53 mutational spectrum in ovarian carcinomas." Ann Hum Genet **70**(Pt 5): 594-604.

Davies, J. M., N. Hawe, et al. (1999). "Novel BTB/POZ domain zinc-finger protein, LRF, is a potential target of the LAZ-3/BCL-6 oncogene." Oncogene **18**(2): 365-75.

Davis, R. E., K. D. Brown, et al. (2001). "Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells." J Exp Med **194**(12): 1861-74.

de Fougerolles, A., H. P. Vornlocher, et al. (2007). "Interfering with disease: a progress report on siRNA-based therapeutics." Nat Rev Drug Discov **6**(6): 443-53.

Delabie, J., E. Vandenberghe, et al. (1992). "Histiocyte-rich B-cell lymphoma. A distinct clinicopathologic entity possibly related to lymphocyte predominant Hodgkin's disease, paragranuloma subtype." Am J Surg Pathol **16**(1): 37-48.

DiGiuseppe, J. A., M. S. Redston, et al. (1995). "p53-independent expression of the cyclin-dependent kinase inhibitor p21 in pancreatic carcinoma." Am J Pathol **147**(4): 884-8.

Dogan, A. and P. G. Isaacson (2003). "Splenic marginal zone lymphoma." Semin Diagn Pathol **20**(2): 121-7.

Donehower, L. A., M. Harvey, et al. (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." Nature **356**(6366): 215-21.

Dryver, E., L. Brandt, et al. (2004). "Occupational exposures and non-Hodgkin's lymphoma in Southern Sweden." Int J Occup Environ Health **10**(1): 13-21.

Eguchi, K., T. Yao, et al. (2000). "Discordance of p53 mutations of synchronous colorectal carcinomas." Mod Pathol **13**(2): 131-9.

Elliott, M. J., Y. B. Dong, et al. (2001). "E2F-1 up-regulates c-Myc and p14(ARF) and induces apoptosis in colon cancer cells." Clin Cancer Res **7**(11): 3590-7.

Engel, I. and S. M. Hedrick (1988). "Site-directed mutations in the VDJ junctional region of a T cell receptor beta chain cause changes in antigenic peptide recognition." Cell **54**(4): 473-84.

Erlacher, M., E. M. Michalak, et al. (2005). "BH3-only proteins Puma and Bim are rate-limiting for gamma-radiation- and glucocorticoid-induced apoptosis of lymphoid cells in vivo." Blood **106**(13): 4131-8.

Faneyte, I. F., J. G. Schrama, et al. (2003). "Breast cancer response to neoadjuvant chemotherapy: predictive markers and relation with outcome." Br J Cancer **88**(3): 406-12.

Feng, Y. Z., T. Shiozawa, et al. (2005). "Intratumoral heterogeneous expression of p53 correlates with p53 mutation, Ki-67, and cyclin A expression in endometrioid-type endometrial adenocarcinomas." Virchows Arch **447**(5): 816-22.

Ferlay, J., P. Autier, et al. (2007). "Estimates of the cancer incidence and mortality in Europe in 2006." Ann Oncol **18**(3): 581-92.

Flores, E. R., K. Y. Tsai, et al. (2002). "p63 and p73 are required for p53-dependent apoptosis in response to DNA damage." Nature **416**(6880): 560-4.

Foroutan, B., A. Ali Ruf, et al. (2007). "In vitro studies of DNA damage and its repair in cells from NHL patients with different p53 mutant protein status, resistant (p53(+)) and sensitive (p53(-)) to cancer chemotherapy." J Pharmacol Toxicol Methods **55**(1): 58-64.

Frenzel, A., F. Grespi, et al. (2009). "Bcl2 family proteins in carcinogenesis and the treatment of cancer." Apoptosis **14**(4): 584-96.

Furihata, M., A. Kurabayashl, et al. (2002). "Frequent phosphorylation at serine 392 in overexpressed p53 protein due to missense mutation in carcinoma of the urinary tract." J Pathol **197**(1): 82-8.

Gasser, R. B., M. Hu, et al. (2006). "Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation." Nat Protoc **1**(6): 3121-8.

Girlando, S., P. Slomp, et al. (1999). "p21 expression in colorectal carcinomas: a study on 103 cases with analysis of p53 gene mutation/expression and clinic-pathological correlations." Virchows Arch **435**(6): 559-65.

Goan, Y. G., H. C. Chang, et al. (2005). "Risk of p53 gene mutation in esophageal squamous cell carcinoma and habit of betel quid chewing in Taiwanese." Cancer Sci **96**(11): 758-65.

Gregory, B. D., B. E. Nickels, et al. (2004). "A regulator that inhibits transcription by targeting an intersubunit interaction of the RNA polymerase holoenzyme." Proc Natl Acad Sci U S A **101**(13): 4554-9.

Greiner, A., A. Marx, et al. (1994). "Idiotype identity in a MALT-type lymphoma and B cells in Helicobacter pylori associated chronic gastritis." Lab Invest **70**(4): 572-8.

Gwosdz, C., V. Balz, et al. (2005). "p53, p63 and p73 expression in squamous cell carcinomas of the head and neck and their response to cisplatin exposure." Adv Otorhinolaryngol **62**: 58-71. Habermann, T. M., E. A. Weller, et al. (2006). "Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma." J Clin Oncol **24**(19): 3121-7.

Hainaut, P., T. Soussi, et al. (1997). "Database of p53 gene somatic mutations in human tumors and cell lines: updated compilation and future prospects." Nucleic Acids Res **25**(1): 151-7.

Hainaut, P., T. Hernandez, et al. (1998). "IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools." Nucleic Acids Res **26**(1): 205-13.

Hall, P. A., D. A. Levison, et al. (1990). "Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms." J Pathol **162**(4): 285-94.

Hall, S. R., L. E. Campbell, et al. (1996). "Phosphorylation of p53 at the casein kinase II site selectively regulates p53-dependent transcriptional repression but not transactivation." Nucleic Acids Res **24**(6): 1119-26.

Hao, X. P., T. Gunther, et al. (1998). "Expression of mdm2 and p53 in epithelial neoplasms of the colorectum." Mol Pathol **51**(1): 26-9.

Harris, C. C. (1996). "Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies." J Natl Cancer Inst **88**(20): 1442-55.

Harris, N. L. (1997). "Principles of the revised European-American Lymphoma Classification (from the International Lymphoma Study Group)." Ann Oncol **8 Suppl 2**: 11-6.

Harris, N. L., E. S. Jaffe, et al. (2000). "Lymphoma classification--from controversy to consensus: the R.E.A.L. and WHO Classification of lymphoid neoplasms." Ann Oncol **11 Suppl 1**: 3-10.

He, X., L. He, et al. (2007). "The guardian's little helper: microRNAs in the p53 tumor suppressor network." Cancer Res **67**(23): 11099-101.

He, L., X. He, et al. (2007). "microRNAs join the p53 network--another piece in the tumour-suppression puzzle." Nat Rev Cancer **7**(11): 819-22.

Henriksson, E., B. Baldetorp, et al. (2006). "p53 mutation and cyclin D1 amplification correlate with cisplatin sensitivity in xenografted human squamous cell carcinomas from head and neck." Acta Oncol **45**(3): 300-5.

Hermeking, H. (2007). "p53 enters the microRNA world." Cancer Cell **12**(5): 414-8.

Hermine, O., C. Haioun, et al. (1996). "Prognostic significance of bcl-2 protein expression in aggressive non-Hodgkin's lymphoma. Groupe d'Etude des Lymphomes de l'Adulte (GELA)." Blood **87**(1): 265-72.

Hernandez-Boussard, T., P. Rodriguez-Tome, et al. (1999). "IARC p53 mutation database: a relational database to compile and analyze p53 mutations in human tumors and cell lines. International Agency for Research on Cancer." Hum Mutat **14**(1): 1-8.

Herrinton, L. J. (1998). "Epidemiology of the Revised European-American Lymphoma Classification subtypes." Epidemiol Rev **20**(2): 187-203.

Hinnis, A. R., J. C. Luckett, et al. (2007). "Survivin is an independent predictor of short-term survival in poor prognostic breast cancer patients." Br J Cancer **96**(4): 639-45.

Hirose, Y., Y. Masaki, et al. (2003). "Epstein-Barr virus-associated anaplastic large cell variant of diffuse large B-cell-type non-Hodgkin's lymphoma with concurrent p53 protein expression." Int J Hematol **77**(5): 499-502.

Hjerrild, M., D. Milne, et al. (2001). "Phosphorylation of murine double minute clone 2 (MDM2) protein at serine-267 by protein kinase CK2 in vitro and in cultured cells." Biochem J **355**(Pt 2): 347-56.

Honda, R. and H. Yasuda (1999). "Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53." EMBO J **18**(1): 22-7.

Hoollstein M, S. D., Vogelstein B, Harris CC. (1991). "p53 mutations in human cancers." Science;**253:** : 49-53.

Huang, Y., K. Jin, et al. (2001). "[p53 mutation in lung cancer in Gejiu and Xuanwei regions of Yunnan Province]." Hua Xi Yi Ke Da Xue Xue Bao **32**(3): 361-4.

Huebner-Chan, D., B. Fernandes, et al. (2001). "An immunophenotypic and molecular study of primary large B-cell lymphoma of bone." Mod Pathol **14**(10): 1000-7.

Huuhtanen, R. L., C. P. Blomqvist, et al. (1999). "Comparison of the Ki-67 score and S-phase fraction as prognostic variables in soft-tissue sarcoma." Br J Cancer **79**(5-6): 945-51.

Ichikawa, A., T. Kinoshita, et al. (1997). "Mutations of the p53 gene as a prognostic factor in aggressive B-cell lymphoma." N Engl J Med **337**(8): 529-34.

Ichikawa, A. (2000). "Prognostic and predictive significance of p53 mutation in aggressive B-cell lymphoma." Int J Hematol **71**(3): 211-20.

Iinuma, H., K. Okinaga, et al. (2000). "Detection of tumor cells in blood using CD45 magnetic cell separation followed by nested mutant allele-specific amplification of p53 and K-ras genes in patients with colorectal cancer." Int J Cancer **89**(4): 337-44.

Ivanovska, I., A. S. Ball, et al. (2008). "MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression." Mol Cell Biol **28**(7): 2167-74.

Iwakuma, T., G. Lozano, et al. (2005). "Li-Fraumeni syndrome: a p53 family affair." Cell Cycle **4**(7): 865-7.

Iqbal, J., V. T. Neppalli, et al. (2006). "BCL2 expression is a prognostic marker for the activated B-cell-like type of diffuse large B-cell lymphoma." J Clin Oncol **24**(6): 961-8.

Jacobs, T. W., J. E. Prioleau, et al. (1996). "Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer." J Natl Cancer Inst **88**(15): 1054-9.

Jaffe ES, H. N., Stein H et al., (2001). "Tumours of Haematopoietic and Lymphoid Tissues." eds. Lyon: IARC Press: 171-174.

Janumyan, Y. M., C. G. Sansam, et al. (2003). "Bcl-xL/Bcl-2 coordinately regulates apoptosis, cell cycle arrest and cell cycle entry." EMBO J **22**(20): 5459-70.

Jenkins, L. M., H. Yamaguchi, et al. (2009). "Two Distinct Motifs within the p53 Transactivation Domain Bind to the Taz2 Domain of p300 and Are Differentially Affected by Phosphorylation (dagger)." Biochemistry.

Jerkeman, M., H. Anderson, et al. (2004). "Assessment of biological prognostic factors provides clinically relevant information in patients with diffuse large B-cell lymphoma--a Nordic Lymphoma Group study." Ann Hematol **83**(7): 414-9.

Jeyaraj, S., D. M. O'Brien, et al. (2009). "MDM2 and MDM4 splicing: an integral part of the cancer spliceome." Front Biosci **14**: 2647-56.

Kamata, H., S. Mitani, et al. (2007). "Mutation of the p53 tumour suppressor gene and overexpression of its protein in 62 Japanese non-Hodgkin's lymphomas." Clin Exp Med **7**(2): 39-46.

Kaghad, M., H. Bonnet, et al. (1997). "Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers." Cell **90**(4): 809-19.

Kato, S., A. Shimada, et al. (1999). "Effects of p51/p63 missense mutations on transcriptional activities of p53 downstream gene promoters." Cancer Res **59**(23): 5908-11.

Kelsey, K. T., T. Hirao, et al. (2005). "TP53 alterations and patterns of carcinogen exposure in a U.S. population-based study of bladder cancer." Int J Cancer **117**(3): 370-5.

Kerbauy, F. R., G. W. Colleoni, et al. (2004). "Detection and possible prognostic relevance of p53 gene mutations in diffuse large B-cell lymphoma. An analysis of 51 cases and review of the literature." Leuk Lymphoma **45**(10): 2071-8.

Kern, S. E., K. W. Kinzler, et al. (1991). "Mutant p53 proteins bind DNA abnormally in vitro." Oncogene **6**(1): 131-6.

Kern, S. E., K. W. Kinzler, et al. (1991). "Identification of p53 as a sequence-specific DNA-binding protein." Science **252**(5013): 1708-11.

Kim, E. and W. Deppert (2004). "Transcriptional activities of mutant p53: when mutations are more than a loss." J Cell Biochem **93**(5): 878-86.

Kim, V. N. (2005). "MicroRNA biogenesis: coordinated cropping and dicing." Nat Rev Mol Cell Biol **6**(5): 376-85.

Kim, Y. K., J. Yu, et al. (2009). "Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer." Nucleic Acids Res.

Kindblom, L. G., M. Ahlden, et al. (1995). "Immunohistochemical and molecular analysis of p53, MDM2, proliferating cell nuclear antigen and Ki67 in benign and malignant peripheral nerve sheath tumours." Virchows Arch **427**(1): 19-26.

Klumb, C. E., D. R. Furtado, et al. (2003). "DNA sequence profile of TP53 gene mutations in childhood B-cell non-Hodgkin's lymphomas: prognostic implications." Eur J Haematol **71**(2): 81-90.

Klumb, C. E., R. Hassan, et al. (2004). "p53 protein expression does not correlate with EBV status in childhood B non-Hodgkin lymphomas." Pediatr Blood Cancer **43**(2): 115-9.

Kocialkowski, S., F. Pezzella, et al. (1995). "Mutations in the p53 gene are not limited to classic 'hot spots' and are not predictive of p53 protein expression in high-grade non-Hodgkin's lymphoma." Br J Haematol **89**(1): 55-60.

Kulikov, R., M. Winter, et al. (2006). "Binding of p53 to the central domain of Mdm2 is regulated by phosphorylation." J Biol Chem **281**(39): 28575-83.

Kumamoto, K., E. A. Spillare, et al. (2008). "Nutlin-3a activates p53 to both down-regulate inhibitor of growth 2 and up-regulate mir-34a, mir-34b, and mir-34c expression, and induce senescence." Cancer Res **68**(9): 3193-203.

Kuppers, R., U. Klein, et al. (1999). "Cellular origin of human B-cell lymphomas." N Engl J Med **341**(20): 1520-9.

Kuribayashi, K. and W. S. El-Deiry (2008). "Regulation of programmed cell death by the p53 pathway." Adv Exp Med Biol **615**: 201-21.

Lahav, G., N. Rosenfeld, et al. (2004). "Dynamics of the p53-Mdm2 feedback loop in individual cells." Nat Genet **36**(2): 147-50.

Lane, D. P. and L. V. Crawford (1979). "T antigen is bound to a host protein in SV40-transformed cells." Nature **278**(5701): 261-3.

Lang, G. A., T. Iwakuma, et al. (2004). "Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome." Cell **119**(6): 861-72.

Lau, N. C., A. G. Seto, et al. (2006). "Characterization of the piRNA complex from rat testes." Science **313**(5785): 363-7.

Lee, M. H. and G. Lozano (2006). "Regulation of the p53-MDM2 pathway by 14-3-3 sigma and other proteins." Semin Cancer Biol **16**(3): 225-34.

Lee, Y. I., S. Lee, et al. (2000). "Activation of the insulin-like growth factor II transcription by aflatoxin B1 induced p53 mutant 249 is caused by activation of transcription complexes; implications for a gain-of-function during the formation of hepatocellular carcinoma." Oncogene **19**(33): 3717-26.

Lee, Y. S. and A. Dutta (2006). "MicroRNAs: small but potent oncogenes or tumor suppressors." Curr Opin Investig Drugs **7**(6): 560-4.

le Sage, C. and R. Agami (2006). "Immense promises for tiny molecules: uncovering miRNA functions." Cell Cycle **5**(13): 1415-21.

Levine, A. J., W. Hu, et al. (2006). "The P53 pathway: what questions remain to be explored?" Cell Death Differ **13**(6): 1027-36.

Lewis, B. P., C. B. Burge, et al. (2005). "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets." Cell **120**(1): 15-20.

Li, F. P. (1990). "Familial cancer syndromes and clusters." Curr Probl Cancer **14**(2): 73-114.

Li, M., J. Luo, et al. (2002). "Acetylation of p53 inhibits its ubiquitination by Mdm2." J Biol Chem **277**(52): 50607-11.

Lin, J., A. K. Teresky, et al. (1995). "Two critical hydrophobic amino acids in the N-terminal domain of the p53 protein are required for the gain of function phenotypes of human p53 mutants." Oncogene **10**(12): 2387-90.

Linzer, D. I., W. Maltzman, et al. (1979). "The SV40 A gene product is required for the production of a 54,000 MW cellular tumor antigen." Virology **98**(2): 308-18.

Lo Coco, F., G. Gaidano, et al. (1993). "p53 mutations are associated with histologic transformation of follicular lymphoma." Blood **82**(8): 2289-95.

Lossos, I. S., A. A. Alizadeh, et al. (2000). "Ongoing immunoglobulin somatic mutation in germinal center B cell-like but not in activated B cell-like diffuse large cell lymphomas." Proc Natl Acad Sci U S A **97**(18): 10209-13.

Lu, H., Y. Taya, et al. (1998). "Ultraviolet radiation, but not gamma radiation or etoposide-induced DNA damage, results in the phosphorylation of the murine p53 protein at serine-389." Proc Natl Acad Sci U S A **95**(11): 6399-402.

MacLennan, I. C. (1994). "Germinal centers." Annu Rev Immunol **12**: 117-39.

Marcel, V. and P. Hainaut (2009). "p53 isoforms - a conspiracy to kidnap p53 tumor suppressor activity?" Cell Mol Life Sci **66**(3): 391-406.

Margalit, O., H. Amram, et al. (2006). "BCL6 is regulated by p53 through a response element frequently disrupted in B-cell non-Hodgkin lymphoma." Blood **107**(4): 1599-607.

Marin, M. C., C. A. Jost, et al. (2000). "A common polymorphism acts as an intragenic modifier of mutant p53 behaviour." Nat Genet **25**(1): 47-54.

Matsumoto, M., M. Furihata, et al. (2004). "Prognostic significance of serine 392 phosphorylation in overexpressed p53 protein in human esophageal squamous cell carcinoma." Oncology **67**(2): 143-50.

Mead, J. H. and T. E. Mason (1983). "Lymphoma versus AIDS." Am J Clin Pathol **80**(4): 546-7.

Meek, D. W. (1994). "Post-translational modification of p53." Semin Cancer Biol **5**(3): 203-10.

Miles, R. R., M. Raphael, et al. (2008). "Pediatric diffuse large B-cell lymphoma demonstrates a high proliferation index, frequent c-Myc protein expression, and a high incidence of germinal center subtype: Report of the French-American-British (FAB) international study group." Pediatr Blood Cancer **51**(3): 369-74.

Miller, C., T. Mohandas, et al. (1986). "Human p53 gene localized to short arm of chromosome 17." Nature **319**(6056): 783-4.

Minamoto, T., T. Buschmann, et al. (2001). "Distinct pattern of p53 phosphorylation in human tumors." Oncogene **20**(26): 3341-7.

Miyashita, T., M. Harigai, et al. (1994). "Identification of a p53-dependent negative response element in the bcl-2 gene." Cancer Res **54**(12): 3131-5.

Moller, M. B., Y. Ino, et al. (1999). "Aberrations of the p53 pathway components p53, MDM2 and CDKN2A appear independent in diffuse large B cell lymphoma." Leukemia **13**(3): 453-9.

Moller, M. B., O. Nielsen, et al. (2002). "Frequent alteration of MDM2 and p53 in the molecular progression of recurring non-Hodgkin's lymphoma." Histopathology **41**(4): 322-30.

Mottet, D., S. Pirotte, et al. (2009). "HDAC4 represses p21(WAF1/Cip1) expression in human cancer cells through a Sp1-dependent, p53-independent mechanism." Oncogene **28**(2): 243-56.

Nakamizo, A., T. Amano, et al. (2008). "Phosphorylation of Thr18 and Ser20 of p53 in Ad-p53-induced apoptosis." Neuro Oncol **10**(3): 275-91.

Natkunam, Y., I. S. Lossos, et al. (2005). "Expression of the human germinal center-associated lymphoma (HGAL) protein, a new marker of germinal center B-cell derivation." Blood **105**(10): 3979-86.

Nakamura, S., K. Akazawa, et al. (1996). "Inverse correlation between the expression of bcl-2 and p53 proteins in primary gastric lymphoma." Hum Pathol **27**(3): 225-33.

Nakamura, Y. (2000). "Internal deletions within the BCL6 gene in B-cell non-Hodgkin's lymphoma." Leuk Lymphoma **38**(5-6): 505-12.

Oettinger, M. A. (1992). "Activation of V(D)J recombination by RAG1 and RAG2." Trends Genet **8**(12): 413-6.

Offersen, B. V., J. Alsner, et al. (2008). "A comparison among HER2, TP53, PAI-1, angiogenesis, and proliferation activity as prognostic variables in tumours from 408 patients diagnosed with early breast cancer." Acta Oncol **47**(4): 618-32.

Oka, T., A. B. Sarker, et al. (1998). "p53 protein expression in non-Hodgkin's lymphomas is infrequently related to p53 gene mutations." Pathol Int **48**(1): 15-21.

Olapade-Olaopa, E. O., E. H. MacKay, et al. (1998). "Variability of immunohistochemical reactivity on stored paraffin slides." J Clin Pathol **51**(12): 943.

Oliner, J. D., J. A. Pietenpol, et al. (1993). "Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53." Nature **362**(6423): 857-60.

Ott, K., H. Vogelsang, et al. (2003). "Chromosomal instability rather than p53 mutation is associated with response to neoadjuvant cisplatin-based chemotherapy in gastric carcinoma." Clin Cancer Res **9**(6): 2307-15.

Oudejans, J. J., W. N. van Wieringen, et al. (2009). "Identification of genes putatively involved in the pathogenesis of diffuse large B-cell lymphomas by integrative genomics." Genes Chromosomes Cancer **48**(3): 250-60.

Papadaki, T., K. Stamatopoulos, et al. (2007). "Splenic marginal-zone lymphoma: one or more entities? A histologic, immunohistochemical, and molecular study of 42 cases." Am J Surg Pathol **31**(3): 438-46.

Papp, T., H. Pemsel, et al. (2003). "Mutational analysis of N-ras, p53, CDKN2A (p16(INK4a)), p14(ARF), CDK4, and MC1R genes in human dysplastic melanocytic naevi." J Med Genet **40**(2): E14.

Parant, J. M., V. Reinke, et al. (2001). "Organization, expression, and localization of the murine mdmx gene and pseudogene." Gene **270**(1-2): 277-83.

Parekh, S., G. Prive, et al. (2008). "Therapeutic targeting of the BCL6 oncogene for diffuse large B-cell lymphomas." Leuk Lymphoma **49**(5): 874-82.

Paris, R., R. E. Henry, et al. (2008). "Multiple p53-independent gene silencing mechanisms define the cellular response to p53 activation." Cell Cycle **7**(15): 2427-33.

Peller, S. and V. Rotter (2003). "TP53 in hematological cancer: low incidence of mutations with significant clinical relevance." Hum Mutat **21**(3): 277-84.

Pescarmona, E., P. Pignoloni, et al. (2001). "p53 over-expression identifies a subset of nodal peripheral T-cell lymphomas with a distinctive biological profile and poor clinical outcome." J Pathol **195**(3): 361-6.

Petrocca, F., R. Visone, et al. (2008). "E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer." Cancer Cell **13**(3): 272-86.

Phan, R. T. and R. Dalla-Favera (2004). "The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells." Nature **432**(7017): 635-9.

Pignon, J. M., I. Vinatier, et al. (1994). "Exhaustive analysis of the P53 gene coding sequence by denaturing gradient gel electrophoresis: application to the detection of point mutations in acute leukemias." Hum Mutat **3**(2): 126-32.

Pivnick, E. K., W. L. Furman, et al. (1998). "Simultaneous adrenocortical carcinoma and ganglioneuroblastoma in a child with Turner syndrome and germline p53 mutation." J Med Genet **35**(4): 328-32.

Prioleau, J. and S. J. Schnitt (1995). "p53 antigen loss in stored paraffin slides." N Engl J Med **332**(22): 1521-2.

Qing Ji,[2] Xinbao Hao, Yang Meng, Min Zhang, Jeffrey DeSano, Daiming Fan, and Liang Xu (2008). "Restoration of tumor suppressor miR-34 inhibits human p53-mutant gastric cancer tumorspheres." BMC Cancer. 8: 266.

Qu, L., S. Huang, et al. (2004). "Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3beta." Genes Dev **18**(3): 261-77.

Quesnel, S., S. Verselis, et al. (1999). "p53 compound heterozygosity in a severely affected child with Li-Fraumeni syndrome." Oncogene **18**(27): 3970-8.

Rafaniello Raviele, P., G. Pruneri, et al. (2009). "Plasmablastic lymphoma: a review." Oral Dis **15**(1): 38-45.

Raible, M. D., E. D. Hsi, et al. (1999). "Bcl-6 protein expression by follicle center lymphomas. A marker for differentiating follicle center lymphomas from other low-grade lymphoproliferative disorders." Am J Clin Pathol **112**(1): 101-7.

Ramsay, A. D., W. J. Smith, et al. (1988). "T-cell-rich B-cell lymphoma." Am J Surg Pathol **12**(6): 433-43.

Rao, S., M. Lowe, et al. (1998). "Lovastatin mediated G1 arrest in normal and tumor breast cells is through inhibition of CDK2 activity and redistribution of p21 and p27, independent of p53." Oncogene **17**(18): 2393-402.

Rappaport, H., Ed. (1966). Atlas of Tumor Pathology. Tumors of the hematopoietic system. . Washington, DC.

Raver-Shapira, N. and M. Oren (2007). "Tiny actors, great roles: microRNAs in p53's service." Cell Cycle **6**(21): 2656-61.

Rosenberg SA, B. C., Brown B et al (1982). "National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: summary and description of a working formulation for clinical usage. The Non-Hodgkin's Lymphoma Pathologic Classification Project." Cancer **49**(10): 2112-35.

Rothman, N., C. F. Skibola, et al. (2006). "Genetic variation in TNF and IL10 and risk of non-Hodgkin lymphoma: a report from the InterLymph Consortium." Lancet Oncol **7**(1): 27-38.

Sanchez-Aguilera, A., J. F. Garcia, et al. (2006). "Hodgkin's lymphoma cells express alternatively spliced forms of HDM2 with multiple effects on cell cycle control." Oncogene **25**(18): 2565-74.

Saumet, A. and C. H. Lecellier (2006). "Anti-viral RNA silencing: do we look like plants?" Retrovirology **3**: 3.

Sahu, G. R. and B. R. Das (2002). "Prognostic significance of p53 and Bcl-2 in acute lymphoblastic leukemia." Oncol Rep **9**(6): 1391-8.

Sanchez-Beato, M., A. I. Saez, et al. (2001). "Overall survival in aggressive B-cell lymphomas is dependent on the accumulation of alterations in p53, p16, and p27." Am J Pathol **159**(1): 205-13.

Sanchez-Beato, M., A. Sanchez-Aguilera, et al. (2003). "Cell cycle deregulation in B-cell lymphomas." Blood **101**(4): 1220-35.

Sehn, L. H., J. Donaldson, et al. (2005). "Introduction of combined CHOP plus rituximab therapy dramatically improved outcome of diffuse large B-cell lymphoma in British Columbia." J Clin Oncol **23**(22): 5027-33.

Sen, C. K. and S. Roy (2007). "miRNA: licensed to kill the messenger." DNA Cell Biol **26**(4): 193-4.

Sheikh, M. S. and A. J. Fornace, Jr. (1999). "Regulation of translation initiation following stress." Oncogene **18**(45): 6121-8.

Sherr, C. J. (1993). "Mammalian G1 cyclins." Cell **73**(6): 1059-65.

Sigal, A. and V. Rotter (2000). "Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome." Cancer Res **60**(24): 6788-93.

Skinnider, B. F., D. E. Horsman, et al. (1999). "Bcl-6 and Bcl-2 protein expression in diffuse large B-cell lymphoma and follicular lymphoma: correlation with 3q27 and 18q21 chromosomal abnormalities." Hum Pathol **30**(7): 803-8.

Soong, R. and B. J. Iacopetta (1997). "A rapid and nonisotopic method for the screening and sequencing of p53 gene mutations in formalin-fixed, paraffin-embedded tumors." Mod Pathol **10**(3): 252-8.

Soussi, T. and C. Beroud (2001). "Assessing TP53 status in human tumours to evaluate clinical outcome." Nat Rev Cancer **1**(3): 233-40.

Stokke, T., E. Galteland, et al. (2000). "Oncogenic aberrations in the p53 pathway are associated with a high S phase fraction and poor patient survival in B-cell Non-Hodgkin's lymphoma." Int J Cancer **89**(4): 313-24.

Stansfeld, A. G., J. Diebold, et al. (1988). "Updated Kiel classification for lymphomas." Lancet **1**(8580): 292-3.

Sturm, I., A. G. Bosanquet, et al. (2003). "Mutation of p53 and consecutive selective drug resistance in B-CLL occurs as a consequence of prior DNA-damaging chemotherapy." Cell Death Differ **10**(4): 477-84.

Taylor, W. R. and G. R. Stark (2001). "Regulation of the G2/M transition by p53." Oncogene **20**(15): 1803-15.

ten Berge, R. L., J. J. Oudejans, et al. (2003). "ALK-negative systemic anaplastic large cell lymphoma: differential diagnostic and prognostic aspects--a review." J Pathol **200**(1): 4-15.

Tannapfel, A., C. Busse, et al. (2001). "INK4a-ARF alterations and p53 mutations in hepatocellular carcinomas." Oncogene **20**(48): 7104-9.

Tarasov, V., P. Jung, et al. (2007). "Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest." Cell Cycle **6**(13): 1586-93.

Tokalov, S. V., S. Pieck, et al. (2007). "Varying responses of human cells with discrepant p53 activity to ionizing radiation and heat shock exposure." Cell Prolif **40**(1): 24-37.

Toledo, F. and G. M. Wahl (2006). "Regulating the p53 pathway: in vitro hypotheses, in vivo veritas." Nat Rev Cancer **6**(12): 909-23.

Unger, T., T. Juven-Gershon, et al. (1999). "Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2." EMBO J **18**(7): 1805-14.

van den Broek, L. J. and M. J. van de Vijver (2000). "Assessment of problems in diagnostic and research immunohistochemistry associated with epitope instability in stored paraffin sections." Appl Immunohistochem Mol Morphol **8**(4): 316-21.

Villuendas, R., F. Pezzella, et al. (1997). "p21WAF1/CIP1 and MDM2 expression in non-Hodgkin's lymphoma and their relationship to p53 status: a p53+, MDM2-, p21-immunophenotype associated with missense p53 mutations." J Pathol **181**(1): 51-61.

Villuendas, R., M. A. Piris, et al. (1992). "P53 protein expression in lymphomas and reactive lymphoid tissue." J Pathol **166**(3): 235-41.

Visco, C., F. Canal, et al. (2006). "The impact of P53 and P21(waf1) expression on the survival of patients with the germinal center phenotype of diffuse large B-cell lymphoma." Haematologica **91**(5): 687-90.

Vogelstein, B. and K. W. Kinzler (1992). "p53 function and dysfunction." Cell **70**(4): 523-6.

Vousden, K. H. (2000). "p53: death star." Cell **103**(5): 691-4.

Walker, K. K. and A. J. Levine (1996). "Identification of a novel p53 functional domain that is necessary for efficient growth suppression." Proc Natl Acad Sci U S A **93**(26): 15335-40.

Walsh, T., S. Casadei, et al. (2006). "Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer." JAMA **295**(12): 1379-88.

Wang, V. and W. Wu (2009). "MicroRNA-based therapeutics for cancer." BioDrugs **23**(1): 15-23.

Wang, Y. and C. Prives (1995). "Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases." Nature **376**(6535): 88-91.

Wei, C. L., Q. Wu, et al. (2006). "A global map of p53 transcription-factor binding sites in the human genome." Cell **124**(1): 207-19.

Went, P., T. Dellas, et al. (2004). "[Expression profile and prognostic significance of CD24, p53 and p21 in lymphomas. A tissue microarray study of over 600 non-Hodgkin lymphomas]." Dtsch Med Wochenschr **129**(40): 2094-9.

Werness, B. A., A. J. Levine, et al. (1990). "Association of human papillomavirus types 16 and 18 E6 proteins with p53." Science **248**(4951): 76-9.

Wilder, R. B., M. A. Rodriguez, et al. (2002). "International prognostic index-based outcomes for diffuse large B-cell lymphomas." Cancer **94**(12): 3083-8.

Wong, P., S. J. Verselis, et al. (2006). "Prevalence of early onset colorectal cancer in 397 patients with classic Li-Fraumeni syndrome." Gastroenterology **130**(1): 73-9.

Wright, G., B. Tan, et al. (2003). "A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma." Proc Natl Acad Sci U S A **100**(17): 9991-6.

Yamakuchi, M., M. Ferlito, et al. (2008). "miR-34a repression of SIRT1 regulates apoptosis." Proc Natl Acad Sci U S A **105**(36): 13421-6.

Yang, A., M. Kaghad, et al. (1998). "p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities." Mol Cell **2**(3): 305-16.

Yap, D. B., J. K. Hsieh, et al. (2004). "Ser392 phosphorylation regulates the oncogenic function of mutant p53." Cancer Res **64**(14): 4749-54.

Zainuddin, N., M. Berglund, et al. (2009). "TP53 mutations predict for poor survival in de novo diffuse large B-cell lymphoma of germinal center subtype." Leuk Res **33**(1): 60-6.

Zhang, A., K. Ohshima, et al. (1999). "Prognostic clinicopathologic factors, including immunologic expression in diffuse large B-cell lymphomas." Pathol Int **49**(12): 1043-52.

Zhang, B., X. Pan, et al. (2007). "microRNAs as oncogenes and tumor suppressors." Dev Biol **302**(1): 1-12.

Zhang, Y. and Y. Xiong (2001). "Control of p53 ubiquitination and nuclear export by MDM2 and ARF." Cell Growth Differ **12**(4): 175-86.

Zhao, H., F. Traganos, et al. (2008). "Phosphorylation of p53 on Ser15 during cell cycle caused by Topo I and Topo II inhibitors in relation to ATM and Chk2 activation." Cell Cycle **7**(19): 3048-55.

Zopf, S., D. Neureiter, et al. (2007). "Differential response of p53 and p21 on HDAC inhibitor-mediated apoptosis in HCT116 colon cancer cells in vitro and in vivo." Int J Oncol **31**(6): 1391-402.

NHS
National Research Ethics Service
Leicestershire, Northamptonshire & Rutland Research Ethics Committee 2
Research Ethics Office
Derwent Shared Services
Laurie House
Colyear Street
DERBY
DE1 1LJ

Tel: 01332 868842
Fax: 01332 868785

23 April 2007

Mr Stefan S Antonowicz
Medical student
Maurice Shock Building,
University of Leicester
PO Box 138
Leicester
LE1 9HN

Dear Mr Antonowicz

| | |
|---|---|
| **Study title:** | **A comparative study of haematological tissues with novel diagnostic antibodies for leukaemia and lymphoma** |
| **REC reference:** | **05/Q2502/145** |
| **Amendment number:** | **Substantial Amendment No. 1 dated 3.1.07** |
| **Amendment date:** | **19 February 2007** |

The above amendment was reviewed at the meeting of the Sub-Committee of the REC held on 19 April 2007.

**Ethical opinion**

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

**Approved documents**

The documents reviewed and approved at the meeting were:

| Document | Version | Date |
|---|---|---|
| Protocol | Version 2 | 19 February 2007 |
| CV | Dalia Azam | 21 February 2007 |
| CV | Dr Pringle | 26 February 2007 |
| Notice of Substantial | Substantial Amendment No. 1 | 19 February 2007 |

| | | |
|---|---|---|
| Amendment (non-CTIMPs) | dated 3.1.07 | |

**R&D approval**

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

| | |
|---|---|
| **05/Q2502/145: correspondence** | **Please quote this number on all** |

Yours sincerely

**Miss Sarah Gill**
**Committee Co-ordinator**

E-mail: sarah.gill@derwentsharedservices.nhs.uk

*Copy to:* *University Hospitals of Leicester NHS Trust*

University Hospitals of Leicester NHS

**DIRECTORATE OF RESEARCH AND DEVELOPMENT** NHS Trust

Director: Professor D Rowbotham
Assistant Director: John Hampton
Co-ordinator: M Chapman
Direct Dial: 0116 258 8246
Fax No: 0116 258 4226
EMail: marlene.chapman@uhl-tr.nhs.uk

**Leicester General Hospital**
Gwendolen Road
Leicester
LE5 4PW

Tel: 0116 2490490
Fax: 0116 2584666
Minicom: 0116 2588188

24 April 2007

Dr Howard Pringle
Department of Cancer Studies and Molecular Medicine
RKSCB
Leicester Royal Infirmary
PO Box 65
Leicester
LE2 7LX

Dear Dr Pringle

**09897** **A comparative study of haematological tissues with novel diagnostic antibodies for leukaemia and lymphoma.**

**LREC Ref: 05/Q2502/145** **MREC Ref:**

**Sponsor** University Hospitals of Leicester NHS Trust

**Funder** Dept Funds

**Please note that Trust Indemnity ceases on: 12/01/2010**

We have now been notified by the Ethical Committee that the proposed amendment to this project listed below has been given ethical approval (please see the attached letter from the Ethical Committee).

Protocol amendment, signed and dated 19/02/07 to change the CI to Dr Howard Pringle and to add Mrs Dalia Abdel Azim to the project, to extend the end date of the project to 12/01/2010. Also to carry out DNA sequence analysis.
Protocol, version 2, dated 19 February 2007
CV, Howard Pringle, signed and dated 26/02/07
GCP proforma, signed and dated 27 June 2006
CV, Dalia Abdel Azim, signed and dated 21/02/07
GCP certificate, dated09 February 2007
Ethics approval letter, dated 23 April 2007

I can therefore now re-confirm the full approval of this project on behalf of the University Hospitals of Leicester NHS Trust.

This approval means that you are fully authorised to proceed with the project, using all the resources which you have declared in your original notification form (and subsequent amendments).

The project continues to be covered by Trust Indemnity, except for those aspects already covered by external indemnity (e.g. ABPI in the case of most drug studies).

We will be requesting annual and final reports on the progress of this project, both on behalf of the Trust and on behalf of the Ethical Committee.

Please make sure if you or other researchers have an honorary contract with the Trust that this stays within date whilst working on the research study.

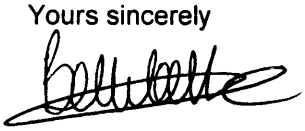
In the meantime, in order to keep our records up to date, could you please notify the Research Office if there are any significant changes to the start or end dates, protocol, funding or costs of the project.

I look forward to the opportunity of reading the published results of your study in due course.

**Below is a list of the Researchers Approved to work on this Application within UHL**

| | |
|---|---|
| Mrs Dalia Abdel Azim | PhD Student |
| Dr Howard Pringle | Chief Investigator (Supervisor) |

Yours sincerely



Elizabeth Kettle

**R&D Manager**