P53 MUTATIONS IN NON-HODGKIN'S LYMPHOMA: CORRELATION WITH PROGNOSIS

by:

- Dorsa Zarrabian-

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Institute of Medical Science
University of Toronto

© Copyright by Dorsa Zarrabian (November 2000)
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-54152-5
P53 MUTATIONS IN NON-HODGKIN'S LYMPHOMA: CORRELATION WITH PROGNOSIS
Master of Science, November 2000
Dorsa Zarrabian
Institute of Medical Science, University of Toronto
Acknowledgments:
Mahinda Samarkoon
Dr. Carol Sawka-Principal Investigator of Biologic Factor Study

ABSTRACT

The most common genetic changes in human tumors are mutations of the p53 gene. The hypothesis of this thesis is that patients with Non-Hodgkin's lymphoma who harbor mutations in the p53 gene have a shorter overall survival compared to patients without p53 mutations. Genomic DNA samples from 134 patients with newly diagnosed Non-Hodgkin's lymphoma were examined for mutations in the p53 gene with the technique of single stranded conformational polymorphism. The presence or absence of p53 mutations was then correlated with clinical and pathologic data.

In total 28 patients were found to have a mutation in the p53 gene. Having a p53 mutation was statistically significant in predicting a shorter overall survival, and also correlated with having an elevated lactate dehydrogenase value. As well, a p53 mutation was nearly correlated with having aggressive disease (intermediate and high grade of the working formulation as opposed to low grade.)
# TABLE OF CONTENTS

LIST OF ABBREVIATIONS vi

GLOSSARY vii

CHAPTER I – INTRODUCTION 1

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>REVIEW OF NON-HODGKIN'S LYMPHOMA 3</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>EPIDEMIOLOGY 3</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>CLASSIFICATION 4</td>
<td></td>
</tr>
<tr>
<td>B (i)</td>
<td>THE WORKING FORMULATION 9</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>STAGING 13</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>TREATMENT OF NHL 14</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>PROGNOSTIC FACTORS 18</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>PATHOGENESIS 21</td>
<td></td>
</tr>
<tr>
<td>D (i)</td>
<td>PRONTO-ONCOGENES 21</td>
<td></td>
</tr>
<tr>
<td>D (ii)</td>
<td>TUMOR SUPPRESSOR GENES 27</td>
<td></td>
</tr>
<tr>
<td>D (iii)</td>
<td>VIRAL INFECTION 27</td>
<td></td>
</tr>
</tbody>
</table>

II THE P53 PROTEIN 29

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>GENE AND PROTEIN STRUCTURE 29</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>BIOLOGIC AND BIOCHEMICAL PROPERTIES 33</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>CELL CYCLE ARREST 37</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>APOPTOSIS 39</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CELL CYCLE ARREST OR APOPTOSIS 41</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>LI-FRAUMENI SYNDROME 41</td>
<td></td>
</tr>
</tbody>
</table>
### FIGURES AND TABLES

#### FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B-Cell Development</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>T-Cell Development</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Schematic Representation of the P53 Molecule</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>Mutant and W.T. SSCP Patterns For Each Condition For Exons 5-8</td>
<td>56</td>
</tr>
<tr>
<td>5</td>
<td>Sensitivity Assay</td>
<td>57</td>
</tr>
</tbody>
</table>

#### TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The Working Formulation</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>The Ann Arbor Staging Classification</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>ECOG Performance Status</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td>Positive and Negative Control Cell Lines Used For Each Exon</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>Conditions Chosen For Detection of P53 Mutations For Each Exon</td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>Mutations Detected</td>
<td>61</td>
</tr>
<tr>
<td>7</td>
<td>Correlations of P53 Mutations With Clinical and Pathologic Data</td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>Pathologic Classification of Patients With and Without P53 Mutations</td>
<td>64</td>
</tr>
<tr>
<td>9</td>
<td>Prevalence of P53 Mutations In Aggressive vs. Low Grade NHL In Other Studies</td>
<td>67</td>
</tr>
<tr>
<td>10</td>
<td>Summary of Studies Looking At Relationship Between P53 Over-expression/Mutation and Survival/Complete Remission/Drug Resistance</td>
<td>77</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

CHOP - Cyclophosphamide, vincristine, doxorubicin, and prednisone
CR - Complete remission
CVP - Cyclophosphamide, vincristine, and prednisolone
DLCL - Diffuse large cell lymphoma
EBV - Epstein Barr virus
ECOG - Eastern Co-operative Oncology Group
FACS - Fluorescence activated cell sorting
HD - Hodgkin's Disease
IFN - Interferon therapy
NHL - Non-Hodgkin's lymphoma
PCNA - Proliferating cell nuclear antigen
RT - Radiotherapy
SSCP - Single strand conformational polymorphism
Tdt - Terminal deoxynucleotidy transferase
GLOSSARY

Retrovirus – A minimum infectious agent consisting of single stranded RNA molecules surrounded by an envelope. The RNA can be reverse transcribed and translated into viral proteins upon infection of the host organism.

Virus - An infectious agent consisting of a nucleic acid (the nucleoid - DNA or RNA, but not both) and a protein shell or capsid, which contains and protects the nucleic acid.

Gene – A segment of DNA capable of being transcribed and translated into a protein in an organism

Protein – A macromolecule made up of a combination of amino acids with an amino and a carboxy end. In humans proteins are made up of combinations of 20 essential amino acids.

Nucleus – The nucleus is the major organelle of eukaryotic cells, in which the chromosomes are separated from the cytoplasm by the nuclear envelope. This organelle distinguishes eukaryotic from prokaryotic cells

Non-Hodgkin’s Lymphoma - A heterogeneous group of diseases consisting of neoplastic proliferation of lymphoid cells.
CHAPTER I - INTRODUCTION

Lymphomas are a heterogeneous group of neoplasms arising in the reticuloendothelial and lymphatic systems. They are divided into the broad groups of Hodgkin's disease (HD), characterized by the presence of multinucleate Reed-Sternberg cells, and Non Hodgkin's Lymphoma (NHL).

NHL encompasses more than a dozen neoplasms of the lymphoid system, and from a clinicopathologic viewpoint can be divided into the broad categories of low, intermediate and high grade. These groupings are based on histology and immunohistochemistry. In the untreated state the low grade lymphomas exhibit an indolent behavior with a median survival of 5 to 10 years, the intermediate lymphomas have a median survival of 2 to 5 years, and the aggressive lymphomas have a six month to two years median survival rate.

The histology, biologic behavior and treatment response of non-Hodgkin's lymphomas are heterogeneous (1). Prognosis and appropriate treatment has been based on accurate grading which is largely dependent on histologic classification (2). Histopathologic interpretation of lymphomas, however, can be subjective, as shown by the marked inter-observer differences in lymphoma grading (3). Some clinical parameters (age, stage, serum lactate dehydrogenase level, extranodal disease, and tumor burden) have been shown to influence survival and also have been used for prognostic and treatment purposes (4, 5).

Many patients with intermediate and aggressive NHL are cured by combination
chemotherapy, however there are those that are not cured and ultimately die of their disease. The use of prognostic factors to identify high and low risk patients with aggressive NHL has important therapeutic implications. High risk patients can be given newer more aggressive regimens, while low risk patients can be treated with standard therapy, thereby avoiding the toxicity associated with more aggressive regimens.

Mutations of the p53 gene are present in at least 50% of all human tumors. The p53 protein is a multifaceted transcription factor which can regulate multiple cellular processes including cell cycle progression, apoptosis, and DNA repair. Thus mutations in the p53 gene are a good candidate as a prognostic factor.

The hypothesis of this thesis is that having a p53 mutation correlates with a shorter overall survival in patients with NHL. The technique of single strand conformational polymorphism was used to detect mutations in genomic DNA samples from 134 newly diagnosed NHL patients. The presence of a p53 mutation was then correlated with clinical and pathologic data.

This thesis is divided into four chapters: Chapter I includes introduction; Non-Hodgkin's Lymphoma will be reviewed, including its classification, pathogenesis, management, and prognostic factors. As well, the p53 gene and protein will be discussed, along with different strategies that can be used to analyze alterations of the p53 gene and protein. Chapter II will discuss Methods. Chapter III will include Results, and Discussion will comprise Chapter IV.
I. REVIEW OF NON-HODGKIN'S LYMPHOMA

A. EPIDEMIOLOGY

Incidence rates for NHL increased by about 50% or more in 20 countries in the last two decades (6). Age-adjusted incidence rates are higher in the developed countries (6). The incidence of NHL varies by age, with the incidence rate increasing exponentially with age between 20 and 79 years (7). Race also affects NHL rates, with whites having higher rates than blacks or Asians (8). The current U.S. age-adjusted incidence rate is 15.1 per 100,000 person-years for both sexes. In the U.S. the incidence of NHL has increased by 73% between 1973 and 1994. The increase has been 3% per year for women and 4% per year for men, with extranodal disease increasing more rapidly than nodal disease (9). A change in the classification of lymphoma, accounting for a 10%-15% shift of HD to NHL could account for only some of the increase in NHL (10). The incidence of high-grade lymphomas has increased more than low-grade lymphomas with diffuse large cell lymphoma showing the greatest increase (11). Mortality rates for NHL have also increased, with mortality rates in the U.S. increasing at 2% per year. Five year survival rates are higher among whites than blacks. Females have a better survival outcome, as do patients younger than 65 years of age (7).

There are several risk factors associated with developing NHL. One risk factor, the Epstein Barr Virus (EBV), is associated with endemic Burkitt’s lymphoma and is present in 100% of cases with chromosome 8 breakpoints usually within the c-myc gene (12). There is also a clear association between immune dysfunction and NHL. As many as 25% of patients with primary immunodeficiencies, such as ataxia telangiectasia, will develop cancers, and NHL accounts for 50% of such cancers (13). Human immunodeficiency virus also increases the risk of developing NHL.
which becomes higher after immunosuppressive therapy (14).

B. CLASSIFICATION

NHL may originate in T cells or B cells. Phenotypic and nodular characteristics of the tumor cells can help to differentiate between the different cells of origin. The majority of NHL (80%-85%) are of B-cell origin, the remainder being T-cell tumors (15). Normal B-cells form follicles within lymph nodes; malignant B-cells mimic this behavior with nodule formation. Nodular lymphomas therefore are always composed of B-cells. Tumors of B and T cells can represent cells arrested at any stage along their differentiation pathway, with the immunophenotype of the different lymphoma subtypes reflecting their state of arrest (Figure 1 and 2). During antigen-induced differentiation within germinal centers of lymph nodes normal B-cells undergo a series of morphologic changes characterized by changes in cell and nuclear size and in nuclear configuration. The changes are as follow: small cleaved to large cleaved cell to small noncleaved cell to large noncleaved cell (16). Nodular lymphomas arising from these follicular center cells are composed of any of these differentiation patterns, as if they had been arrested at a particular stage of differentiation. The general category of B-cell lymphomas includes the small lymphoid B-cell entities, which are typically indolent or low grade, and the B-cell lymphomas with a large or blastic cell component, which are typically intermediate or high grade lymphomas. T-cell Non-Hodgkin’s lymphomas usually have an immunotype corresponding to the post-thymic stage of T-cell development (15).
The Rappaport classification introduced almost fifty years ago, separates NHL into two clinical categories: those that are clustered into identifiable nodules (nodular lymphoma) or spread diffusely throughout the node (diffuse lymphomas). Both patterns destroy the normal lymph architecture (17). In general, nodular (or follicular) architecture is associated with a significantly superior prognosis to that of the diffuse pattern (17, 18). The Working Formulation classification, introduced in 1982, builds on the Rappaport classification by incorporating immunological indices.

The Working Formulation (Table 1) categories include low, intermediate, and high grade lymphoma types, based upon decreasing survival respectively (11). Paradoxically, with the development of chemotherapy regimens the prognosis changed because of the increasing cure rates of patients in the intermediate and high grade lymphomas and the eventual relapse and death of most patients with low grade lymphoma (11). More recently the REAL classification has been introduced which emphasizes the various lymphoma subtypes as disease entities, instead of merely defining the lymphoma by the size, shape, and phenotypes of the cells of which it is comprised (15, 18, 19, 20).
Figure 1 - B Cell Development - B-cell production starts before birth in the fetal liver and continues in the bone marrow. B-lymphocyte maturation is divided into two stages: antigen-independent B-lymphopoiesis, which takes place in the fetal liver and adult bone marrow, and antigen dependent B-cell maturation. The principal cell type involved in humoral immunity are B-lymphocytes. B-effector cells, especially plasma cells, produce immunoglobulins. An immunoglobulin molecule is made up of two heavy chains and two associated identical light chains. Immunoglobulin molecules are antibodies that bind antigen specifically with the N-terminal variable (V) domains of their heavy and light chains. The constant (C) terminal region of the secreted Ig molecules binds to complement components. Resting naive or memory B cells carry cell surface membrane bound Ig(sIg) receptors on their cell surface which are necessary for cellular activation and selection processes during B-cell maturation. They can also bind and internalize soluble antigens for subsequent presentation to T cells. Surface Ig molecules are noncovalently associated with CD79 molecules which are required for a complete signal transduction on antigen receptor engagement. Other accessory molecules that modulate antigen receptor signaling include CD19/CD21, CD22, CD20 and CD40. CD19 and CD21 are noncovalently associated to form a multimolecular signal transduction complex in the B-cell surface. CD22 is a glycoprotein belonging to the Ig superfamily. CD20 and CD40 are membrane proteins. Other B-cell antigenic molecules include ClgM (cytoplasmic IgM), Clg (cytoplasmic Ig), CD10, CD34, & Tdt (terminal deoxynucleotidyl transferase) CD11C, CD25, CD56, CD38, CD5, DR and IgH chain R (reference 21).
Figure 2 – T-Cell Development - T-cells undergo a selection process in the thymus. The T-cell receptor (TCR), which recognizes antigen, allows T-cells to react specifically against exogenous or endogenous antigens. TCRs are disulfide-linked heterodimeric polypeptide molecules primarily consisting of α-β chains. An alternative form of TCR composed of γ-δ chains is found in 5%-10% of mature T lymphocytes. Unlike Ig molecules, TCRs do not recognize soluble antigens, but instead interact with antigens bound to special-presentation molecules encoded by the major histocompatibility complex (MHC). TCR molecules are associated with a number of other surface molecules including CD3, CD4 or CD8, CD2, integrin receptors, CD45, or CD28 molecules. CD3 is noncovalently associated with TCR and is responsible for the signal transduction on antigenic stimulation. Mature T cells express either the CD4 or the CD8 receptor on their surface. CD4-expressing cells typically interact with cells displaying MHC class II (MHC II) and function as helper cells to regulate other cellular responses. CD8 lymphocytes recognize MHC I molecules and are primarily cytotoxic. CD2, which is a glycoprotein, enhances the TCR/CD3 mediated response. CD45 is a transmembrane glycoprotein which acts as a phosphatase. The other cell surface molecules include CD5, CD1, Bcl-2, CD56, CD10, CD30, CD7, Tdt, CD25, CD45, DR. ALL - acute lymphoblastic leukemia. LBL - lymphoblastic lymphoma (reference 21).
TABLE 1
THE WORKING FORMULATION

Low Grade

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Malignant lymphoma small lymphocytic</td>
</tr>
<tr>
<td>B</td>
<td>Malignant lymphoma, follicular, predominantly small cleaved cell</td>
</tr>
<tr>
<td>C</td>
<td>Malignant lymphoma, follicular, mixed, small cleaved and large cell</td>
</tr>
</tbody>
</table>

Intermediate Grade

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>Malignant lymphoma, follicular, predominantly large cell</td>
</tr>
<tr>
<td>E</td>
<td>Malignant lymphoma, diffuse, small cleaved cell</td>
</tr>
<tr>
<td>F</td>
<td>Malignant lymphoma, diffuse, mixed, small and large cell</td>
</tr>
<tr>
<td>G</td>
<td>Malignant lymphoma, diffuse, large cell</td>
</tr>
</tbody>
</table>

High Grade

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>Malignant lymphoma, large cell, immunoblastic</td>
</tr>
<tr>
<td>I</td>
<td>Malignant lymphoma, lymphoblastic</td>
</tr>
<tr>
<td>J</td>
<td>Malignant lymphoma, small noncleaved cell</td>
</tr>
</tbody>
</table>

(Reference 11)
B. (i) THE WORKING FORMULATION

The Working Formulation of Non-Hodgkin’s lymphomas for clinical usage is shown in Table 1.

LOW GRADE—Low grade lymphomas are made up of small lymphocytic lymphoma (SLL), follicular, predominantly small cleaved, and follicular mixed small cleaved and large cell. Small lymphocytic lymphoma consists of small apparently unstimulated lymphocytes with dark-staining round nuclei, scanty cytoplasm, and little variation in size. Mitotic figures are rare (22). The immunophenotype is of mature B cells with surface immunoglobulin, CD19, CD20, CD21, and CD23 expression. They also aberrantly express CD5, CD43, and pan-T antigen (23-25). The normal cellular equivalent of SLL is the CD5+ autoantibody producing B cells at the edge of germinal centers (26).

Follicular lymphoma is the most common subtype of NHL, accounting for 45% of all newly diagnosed patients in the U.S. (11). They are mature B-cell neoplasms with phenotypic and microanatomic features that mimic the germinal centers from which they are derived. Follicular large cell lymphoma is considered intermediate grade in the Working Formulation. The cells of small cleaved follicular lymphoma are slightly larger than normal lymphocytes, with scanty cytoplasm. The most distinctive feature of the tumor cells is their irregular "cleaved" nuclear contour, characterized by prominent clefts and linear infoldings. Large cells can be either large cleaved cells or large noncleaved cells. The nuclei of large cleaved cells are irregular in contour, and indented. When the frequency of large cells exceeds 20% but is less than 50%, the term follicular
mixed small and large cell is used (27-29).

Reflecting their germinal center origin, follicular lymphoma cells typically express pan-B markers (CD19, CD20, CD22) and CD10, and immunoglobulin (Ig) (30-33). However in contrast with the polyclonal Ig phenotype in normal germinal centers, follicular lymphomas display a monoclonal phenotype (31).

**INTERMEDIATE GRADE** - Intermediate grade lymphomas include follicular, predominantly large cell lymphoma. diffuse small cleaved cell lymphoma, diffuse mixed small and large cell lymphoma and diffuse large cell lymphoma. Follicular predominantly large cell lymphoma is an uncommon tumor which represents less than 15% of all follicular NHL. The majority of neoplastic cells are large, with cleaved or noncleaved nuclei. There are numerous mitotic figures. Clinically these tumors may evolve into diffuse large cell lymphomas (27, 28).

Diffuse small cleaved cell lymphoma is composed of small cleaved cells that are morphologically and phenotypically similar to those that are present in the follicular small cleaved cell lymphoma (29). They express sIg, pan B cell antigens, and in many cases CD10 (11, 19). Mantle cell lymphoma is a phenotypically and genetically distinct subgroup of diffuse small cleaved cell lymphoma that arises from the mantle zone of lymphoid follicles rather than from follicular center cells. These tumors express pan-B cell antigens, but they can be immunophenotypically distinguished from follicular center cells by the absence of CD10 and the presence of CD5 antigens (34). Further distinctiveness is conferred by the frequent occurrence of t (15, 18) translocation involving the Bcl-1 locus on chromosome 11, and the IgH locus on chromosome 14. This locus
encodes cyclin D1, a proto-oncogene involved in the regulation of the cell cycle (35).

Diffuse mixed small and large cell lymphoma is an uncommon form of intermediate grade lymphoma. It contains a mixture of small cleaved cells already described, and large cells which are transformed lymphoid cells with nuclei at least twice the size of a small lymphocyte. The nuclei have vesicular chromatin, basophilic cytoplasm, prominent nucleoli and a moderate to high proliferation fraction (29, 36). Large cells may be cleaved or noncleaved. Diffuse large cell lymphoma (DLCL) is the most common of intermediate grade lymphomas and contains predominantly large cleaved or noncleaved cells (11). These two types belong to the spectrum of large cell lymphomas and the distinction between them is difficult. Their clinical and immunophenotypic features are similar to those of high grade large cell immunoblastic lymphoma and will be discussed with that entity.

**HIGH GRADE** - The high grade lymphomas are made up of large cell immunoblastic lymphomas, lymphoblastic lymphoma, and small noncleaved lymphoma. The cells of large immunoblastic lymphomas are four to five times larger than small lymphocytes, having a round or oval nucleus which appears vesicular due to margination of chromatin at the nuclear membrane. There are usually one or two centrally placed prominent nucleoli (29).

Large cell immunoblastic lymphomas and the diffuse large cell and mixed lymphomas comprise 40% of NHL, roughly half being large cell morphology (29, 36). These three diffuse lymphomas are aggressive tumors that are rapidly fatal if untreated. These tumors have a high
growth fraction. Most anticancer agents act on cells that are actively dividing, hence complete remission is achieved in 60%-80% of these patients with intensive combination chemotherapy, and roughly 60% are cured (90, 91, 97). It is difficult for pathologists to make the distinction between large cell and large cell immunoblastic lymphoma reliably and reproducibly. Most aggressive lymphomas show a spectrum in cytologic appearance, and in any given field the predominant cell could be large cell or large cell immunoblastic. The designation of immunoblastic lymphoma is reserved for cases in which more than 90% of the cells have an immunoblastic appearance (29).

Immunophenotypically immunoblastic lymphomas and the related diffuse large cell (DLCL) and mixed lymphomas are heterogeneous. Roughly 80% are of B-cell origin, 15% are T-cell, 4% are null, and 1% are biphenotypic (37, 38). The B-cell diffuse large cell lymphomas express at least one of several pan-B antigens, including CD19, CD20, and CD22. Surface immunoglobulin is found in many but not all B-cell DLCL. DLCL may present challenges because loss of pan-B antigens and pan-T antigens may accompany their transformed status (37, 38).

Lymphoblastic lymphoma (LBL) accounts for 30% of pediatric NHL but is uncommon in adults. The cells of lymphoblastic lymphoma have blastic nuclei with fine dusky chromatin, inconspicuous nucleoli, and scant cytoplasm (39). Eighty-five percent are of immature T-cell phenotype and mimic the cortex of the thymus, with co-expression of T-helper (CD4) and T-cytotoxic antigens (CD8) and strong nuclear co-expression of the thymic-derived nuclear enzyme terminal deoxynucleotidyl transferase (Tdt). The remaining 15% are of immature B-cell phenotype and are histologically indistinguishable from T-LBL, with immunophenotyping being necessary to
differentiate between the two (40, 41).

Small noncleaved cell lymphomas form a group of highly aggressive neoplasms that includes Burkitt’s lymphomas (BL), and Burkitt’s-like lymphomas. They account for 5% or less of NHL in adults but approximately 35% of NHL in children. Burkitt’s lymphomas are high-grade lymphomas that are monomorphic proliferations of intermediate-sized cells with round blastic nuclei and multiple basophilic nucleoli. There is a high mitotic rate with a diffuse starry sky pattern of growth (42, 43). Burkitt’s-like lymphoma is a morphologic variant that shows greater nuclear pleomorphism. The nuclei contain one or two eosinophilic nucleoli with a basophilic vacuolated cytoplasm. This appearance is intermediate between Burkitt’s and large cell (immunoblastic) lymphomas (43). Immunologic findings reflect the likely germinal center origin with pan-B antigen expression, including CD10 and monoclonal Ig expression (42, 44). Burkitt’s lymphomas typically have absent expression of both CD18 and CD45 which are relevant to B-T cell interaction. This results in poor T-cell immunosurveillance and loss of B-cell cohesion leading to ready extranodal spread (44).

C. STAGING

The Ann Arbor staging classification is used for the staging of Non-Hodgkin’s lymphomas (Table 2). Radiographic studies have become the predominant form of staging Non-hodgkin’s lymphomas.
TABLE 2
Ann Arbor Staging Classification

<table>
<thead>
<tr>
<th>Stage</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Involvement of a single lymph node region or of a single extranodal organ or site ($I_e$).</td>
</tr>
<tr>
<td>II</td>
<td>Involvement of $\geq 2$ lymph node regions on the same side of the diaphragm, or localized involvement of an extranodal site or organ ($II_e$) and of $\geq 1$ lymph node regions on the same side of the diaphragm.</td>
</tr>
<tr>
<td>III</td>
<td>Involvement of lymph node regions on both sides of the diaphragm, which may not be accompanied by localized involvement of an extranodal organ or site ($III_e$) or spleen ($III_s$) or both ($III_{es}$).</td>
</tr>
<tr>
<td>IV</td>
<td>Diffuse or disseminated involvement of $\geq 1$ distant extranodal organs with or without associated lymph node involvement.</td>
</tr>
</tbody>
</table>

Fever $>38^\circ$, night sweats, and/or weight loss $>10\%$ of body weight in the 6 months preceding admission are defined as systemic symptoms, and denoted by the suffix B. Patients without B symptoms are denoted by the suffix A.

D. TREATMENT OF NHL

The treatment options for NHL depend on the stage and grade of the disease at the time of presentation. The Ann Arbor staging system is based on the number of lymph node sites, disease above or below the diaphragm, involvement of extranodal sites, and the presence or absence of B symptoms (Table 2).

Indolent nodal lymphomas are localized in 10%-15% of cases. In patients with stage I or II disease, regional radiotherapy (RT) results in long-term control with rates of freedom from relapse of 44% to 47% at 10 years and survival rates of 75% for selected patients younger than 60 years (87, 88). An alternative to RT alone in such patients would be the use of combination chemotherapy with RT. In one study when cyclophosphamide, vincristine, doxorubicin, and prednisone (CHOP)
combined with RT was compared to RT alone in such patients, the recurrence-free survival at 5 years was 64% for combined modality as compared to 37% for RT alone (89). However when cyclophosphamide, vincristine, and prednisolone (CVP) and RT was compared to RT alone no difference in outcome was noted (90). Most of the patients with indolent lymphomas have advanced disease at the time of diagnosis. Patients who are asymptomatic may be treated with a watch and wait approach with no documented change in their survival compared to patients who were treated at the time of diagnosis (91). Therefore treatment of advanced stage III or IV indolent lymphoma can vary from the watch and wait approach to the use of single alkylating agents, combination chemotherapy agents with two to four drugs, and high dose therapy with bone marrow transplant reinfusion. In one study, patients with follicular lymphoma and a low tumor burden were randomized to receive chlorambucil, interferon, or no initial treatment. No differences were present in progression-free or overall survival with a median follow-up of 5 years (92). The choice of treatment must consider many factors, including age, symptoms, extent of disease, co-morbid disease, and follow-up. The use of prognostic factors as identified by the international NHL prognostic project has been successfully applied to the indolent lymphomas (93, 94).

The issue with indolent lymphoma is not attaining remission but maintaining it, and thus various maintenance therapies have been devised (95, 96). One popular maintenance therapy has been interferon (IFN) therapy combined with combination chemotherapy. In one study symptomatic patients with follicular lymphoma and a large tumor burden received six monthly cycles of cyclophosphamide, doxorubicin, teniposide, and prednisolone given alone or concurrently with IFN. Patients with responding disease then received maintenance therapy for 1 year either with CHOP
alone every 2 months, or with IFN. The IFN-treated group had higher response rates (85% vs. 69%), median event-free survival (34 months vs. 19 months), and survival (86% vs. 69%) (96). Recurrence is inevitable for advanced disease. If patients do not respond to the same or similar treatment that they received at diagnosis, then a change to a more intensive regimen such as CHOP may be warranted. The purine analogues have been found to be very active in recurrent follicular NHL (97, 98). A popular combination is the regimen of fludarabine, mitoxantrone, and dexamethasone, which has been shown to have a 47% complete remission (CR) and 47% partial response rate in recurrent and refractory disease (97). Newer approaches such as monoclonal antibodies are also promising, with overall response rates ranging from 40%-60% (99).

Management of apparently localized stage I or II aggressive lymphomas (using diffuse large B-cell lymphoma as a common example), consists of combination chemotherapy with or without regional RT (100-102). The diffuse aggressive lymphomas of adults are a diverse group of diseases which have in common an aggressive clinical behavior leading to rapid clinical deterioration of the patient and, at the same time, sensitivity to chemotherapeutic agents, rendering them curable in approximately 50% of the patients (103). In one study patients with good prognosis stage I or II large-cell lymphoma treated with three cycles of CHOP chemotherapy followed by involved field (IF) irradiation at 4000Gy had 99% CR and 85% long term survival (100). In another study patients with stage I or II disease were given CHOP x 8 or CHOP x 3 plus radiation. The four year survival was 75% for CHOP x 8 and 87% for CHOP x 3 plus RT. As well excess cytotoxicity occurred in patients receiving eight courses of CHOP (101). The CHOP regimen is also used for the treatment of
advanced aggressive NHL (bulky stage II, stage III, or IV) and represented the first major breakthrough in their treatment. Follow-up studies have shown that approximately 30%-40% of patients with advanced disease can have long-term disease-free survival with CHOP therapy (104). In the last twenty years new regimens were developed which added active agents to the cyclophosphamide and doxorubicin backbone such as methotrexate or bleomycin (105). A randomized study comparing CHOP, m-BACOD (methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone), MACOP-B (methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, bleomycin), and Pro MACE-Cyta BOM (prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide, nitrogen mustard, vincristine, procarbazine, cytarabine, bleomycin) found similar 6 year survival rates of 33%, 32%, and 34% respectively. However the CHOP regimen was found to be less toxic than the other regimens (fatal reactions of 1% vs. 3%-6% for the other regimens). After reports of this trial CHOP has once again become the gold standard for newly diagnosed patients with aggressive NHL (105).

Use of prognostic factors (discussed later) allows for identification of good-risk patients who may have a predictive 5-year survival of more than 70% compared with poor-risk patients, and thus allows for more specified risk directed therapies (94). Patients in the poor prognosis category can be given more aggressive therapy, as well as being considered for inclusion in clinical trials evaluating newer therapies. In one study previously untreated poor-prognosis patients were treated with high-dose CHOP and granulocyte stimulating factor support. These patients had a complete remission rate of 86% with 69% of the patients disease-free at a median follow-up time of twenty
months (106). Patients who do not go into complete remission with initial induction therapy or relapse at a later time can be given high-dose chemotherapy and transplantation if they are transplant candidates. Several studies have shown encouraging results using this approach. In one study patients with relapsed aggressive NHL received either high-dose chemotherapy and autologous bone marrow transplantation or additional salvage therapy, with 46% and 12% disease-free at 5 years, respectively (106). Patients who are not transplant candidates can have salvage chemotherapy regimens. Some of the popular regimens are mesna, ifosfamide, mitoxantrone, and etoposide or cyclophosphamide. etoposide, procarboxide, with prednisone. Although these regimens have a CR of 20%-40%, the long-term failure-free survival rates are only 5%-15% (108, 109).

F. PROGNOSTIC FACTORS

Prognostic factors are quite important in NHL as they can identify patients who are likely to benefit from more aggressive therapy. The International Prognostic Factor Project was a large international collaborative study which looked at pretreatment clinical features predictive for overall survival and relapse free survival for patients with aggressive NHL who were treated with a doxorubicin-containing combination chemotherapy regimen (94). Age, elevated LDH, performance status, stage (stage I-II vs. stage III-IV), and number of extranodal disease sites were the clinical features that were independently associated with survival. The relative risk associated with each of the clinical features was comparable. Therefore, an individual patient’s relative risk for death was determined by adding the number of adverse prognostic factors present at diagnosis. Each patient was assigned a score, and four relative-risk categories were determined including low, low-intermediate, high-intermediate, and high risk. The predicted 5-year survival for these groups were
73%, 51%, 43%, and 26% respectively (94). For those patients under age 60 years, a separate age-adjusted model was developed. In the younger patients the clinical features that were independently associated with survival included stage, LDH, and performance status. Based on these three features four risk groups were identified with predicted 5-year survival of 83%, 69%, 46%, and 32%. The strength of this study was its large population base. This index has proven useful in patients with both aggressive (94) and low-grade lymphoma (93) including mantle cell lymphoma (110). However a large number of cases fit into the intermediate risk category, making treatment selection problematic (94). As well, underlying biologic markers of disease were not assessed. Another prognostic model known as the M.D. Anderson Tumor Score incorporates the Ann Arbor stage (stage I-II vs. III-IV), an increased B-2 microglobulin (>3), elevated LDH, presence of constitutional B symptoms, and bulky disease (>7cm). One point is assigned for each adverse prognostic feature with the sum representing a tumor score. In one study of 144 patients who were uniformly treated with CHOP, those patients with a tumor score of 0 to 2 had a time to treatment failure of 83% at three years, compared to 24% for those patients with a tumor score of 3 or more (345). An advantage of this system is that only two prognostic categories of low and high risk exist.

Several groups have looked at immunophenotypic markers as possible prognostic indicators. The lymphocyte homing receptor (LHR, CD44) facilitates the binding of lymphocytes to high endothelial venules and permits the extravasation of lymphocytes into nodal area (112,113). In one study, patients whose tumors had increased levels of CD44 expression also had shorter survival (113). The survival rate at 5 years among patients with high CD44 was 45% versus 74% among patients with low or no CD44 (113). Tumor antigens are recognized in association with MHC
molecules, and the absence of MHC molecules can limit host tumor immunosurveillance. Patients with large cell lymphoma whose tumors lacked HLA-DR had significantly shorter median survival than patients with HLA-DR+ tumors (114). As well loss of immunosurveillance as measured by decreased numbers of CD8+ T-tumor infiltrating lymphocytes has been correlated with shortened survival in B-cell diffuse large cell lymphoma (115). Several studies have looked at cell lineage as a prognostic tool. Although an early study showed conflicting results with B and T cell phenotypes having similar survival outcomes (116), more recent studies looking at a spectrum of histologic subtypes (DLCL and diffuse small cell) have found that a T cell phenotype correlates with decreased survival (117, 118). Also loss of a pan B-antigen (CD22) is associated with a poor prognosis. This is likely due to loss of cohesion, allowing dissemination of tumor cells, and creating distant metastases (38).

Lymphomas may result from oncogenic changes leading to a loss of proliferative control. Altered microanatomic patterns of cell proliferation as measured by Ki-67 may be of prognostic value. The Ki-67 monoclonal antibody detects a nuclear proliferation antigen. In patient groups that were similar in age, stage, tumor burden, LDH, therapy and CR rate, the percentage of Ki-67 + cells was closely correlated with median survival (>60% Ki-67 cells, median survival of 8 months vs. <60%- Ki-67 cells, median survival of 39 months, p <0.003) and was an independent factor in multivariate analysis (119, 120). Several groups have also looked at the level of bcl-2 expression as a possible prognostic indicator and have reported that bcl-2 expression carries an adverse prognosis (5, 121).
F. PATHOGENESIS

Lymphomas arise from a clonal expansion of lymphoid cells that are transformed by the accumulation of genetic lesions. Genetic lesions of NHL involve activation of oncogenes, inactivation of tumor suppressor genes (TSG), and to a lesser extent viral infection. Such changes may occur at different stages during the pathway of lymphocyte differentiation. The requirement for multiple events reflects the fact that normal cells have multiple mechanisms to regulate their growth and differentiation, and several separate changes may be required to bypass these controls (12, 45, 46).

D. (i) PROTO-ONCOGENES

Proto-oncogenes are highly conserved genes which, in normal conditions, promote cell growth and/or cell survival under close control of mitogenic stimuli. Structural lesions of proto-oncogenes convert the physiologic version of these genes into constitutively active pathologic oncogenes. The oncogenes involved in NHL are transcription factors or anti-apoptotic genes which are activated by chromosomal translocations involving the site of the proto-oncogenes on one locus and an antigen receptor locus on the partner chromosome. The most common mechanism of proto-oncogene activation in NHL is represented by deregulation driven by regulatory elements derived from juxtaposed Ig gene loci (12).

BCL-6 - Chromosome alterations affecting band 3q27 occur frequently in B-DLCL (46, 47). Usually there are reciprocal translocations between the 3q27 region and other partner chromosomes. Detailed analysis of the 3q27 region led to the discovery of the bcl-6 gene. The bcl-6 protein acts
as a transcriptional repressor that binds specific DNA sequences and represses transcription from linked promoters (48). High levels of bcl-6 expression are restricted to the B-cell lineage with minimal amounts found in other tissues (49). Expression of bcl-6 within the B-cell compartment is regulated during differentiation, with expression in mature B-cells but not in immature bone marrow precursors, or terminally differentiated cells such as plasma cells. As well bcl-6 expression is topographically restricted to the germinal center among mature B-cells (49). Mice carrying the bcl-6-/- genotype fail to form germinal centers (GC) and have an impaired antigen-specific IgG response, with a high incidence of infectious disease. It is therefore postulated that bcl-6 is needed for germinal center development and its downregulation is necessary for differentiation of B cells into plasma cells or memory B cells (50).

Bcl-6 rearrangements can be detected in 40% of B-DLCL and up to 10% of follicular lymphomas (12). Translocations involving the bcl-6 gene leave the coding domain intact but disrupt the 5' regulatory sequences which contain the promoter (51). A common feature of all promoters linked to the bcl-6 alleles is that they are physiologically active in normal B cells and are not downregulated during the late stages of B cell differentiation (52). Thus downregulation of bcl-6 is prevented, blocking the differentiation of germinal center B cells towards the stage of plasma cells. The role of bcl-6 in B-DLCL development is unclear. One hypothesis is that the continued expression of bcl-6 renders germinal center cells incapable of differentiating further. The normal counterpart of B-DLCL cells is thought to be the proliferating B cells of the dark zone of the germinal center, which lends support to this theory (53).
The heterogeneity of B-DLCL is likely due to its pathogenetic heterogeneity (54). There are at least three molecular pathways in association with B-DLCL. The first molecular type of B-DLCL, accounting for 40% of the cases, arises de novo with no prior history of follicular lymphoma, and displays rearrangements of bcl-6 with no other known genetic alterations of NHL. These cases have the most favourable prognosis (54-56). The second type deriving from the histologic transformation of a follicular lymphoma, have bcl-2 activation, either alone or in association with p53 mutations. These cases have the poorest outcome (54-56). The third type, having germline Bcl-2 and Bcl-6 alleles and no other consistent genetic abnormalities, have an intermediate prognosis (54-56).

C-MYC - Myc is an ubiquitous nuclear protein whose expression is normally tightly controlled. It plays a role in controlling cell proliferation, differentiation, as well as apoptosis (57). Expression of c-myc is higher in proliferating cells and is rapidly induced in quiescent lymphocytes upon mitogenic induction (58). Furthermore, c-myc antisense oligonucleotides prevent activated lymphocytes from entering S phase, thereby inducing growth arrest and differentiation of lymphoid cells (59). However in the absence of adequate concentrations of growth stimuli in the environment, c-myc can induce apoptosis instead of proliferation and instead of block of differentiation (60). Translocations of 8q24, leading to deregulated myc expression provided the first example of the involvement of proto-oncogenes in NHL; they are found in 100% of BL and AIDS-BL, and 30% of AIDS-related DLCL. Three different translocations involving the c-myc gene have been observed (45). The t(8;14) (q24;q32) translocation which involves IgH, occurs in 80% of the cases. In t(2;8) (p11,q24) c-myc juxtaposes to IgK (15% of cases) and to Ig in t(8;22) (q24;11) translocations (5% of cases) (12, 61).
Translocated c-myc genes are juxtaposed to the Ig regulatory elements, leading to constitutive expression of the gene, as would be seen after mitogenic stimulation (61-63). This contrasts with the tight regulation of this gene during normal B cell differentiation and proliferation. The pathogenetic role of c-myc activation in the development of BL is substantiated by in vivo and in vitro studies. Expression of c-myc results in the transformation of EBV-immortalized cell lines (64). The c-myc transgene coupled to the enhancers of the IgM heavy chain or Kappa light chain genes regularly produces aggressive B-cell lymphomas in mice (62). The delay of several months before the focal appearance of lymphomas in transgenic mice and the monoclonality of the tumors are consistent with the operation of other oncogenic events (62). A second oncogenic event may be inactivation of the p53 TSG, which has been shown to be mutated in 30%-40% of BL (12).

BCL-2 - Between 70%-90% of follicular lymphomas and 20% of diffuse B-cell lymphomas have the t (14:18) in which the Bcl-2 gene at 18q21 is juxtaposed to the IgH J segment leading to deregulated bcl-2 expression (65, 66). The bcl-2 gene encodes a 26KD mitochondrial outer membrane protein (67). The protein prevents apoptosis (it protects lymphoid cells against a number of apoptotic stimuli (68)), and prolongs survival (Bcl-2 transfected I1-3 dependent pro-B cells do not undergo apoptosis following I1-3 withdrawal (69)). It has been implicated in normal germinal center physiology (70).

The germinal center is a fundamental component of the humoral immune response, representing a unique microenvironment where antigen activated B lymphocytes undergo clonal expansion, mutate their immunoglobulin, and are subjected to a stringent selection process based on
their antigen affinity (70). There are three histologically distinct microenvironments within the germinal center: a mantle zone, surrounding interior dark and light zones. The mantle zone contains a clonally heterogeneous collection of small intermediate, and blastic lymphoid cells that move into and out of the germinal center. Antigen triggered activation of the B cells of the dark zone drives proliferation along with somatic hypermutation of the Ig variable regions of these cells. B cells that cannot interact with antigen through their Ig receptors, however, undergo apoptosis. Surviving clones from within the dark zone then enter the light zone where proliferation slows and affinity selection predominates. This process allows for the generation of a population of memory B cells that is relatively long-lived and enables a higher affinity secondary response upon re-exposure to antigen (53, 70). In a normal germinal center, bcl-2 expression is restricted to the mantle zone (composed of long-lived re-circulating B cells) and the light zone (71). In contrast to the focal expression of bcl-2 by normal germinal centers, the malignant follicles in malignant lymphoma strongly express the bcl-2 protein (72).

Under physiologic conditions, B cells which are selected by antigen in the germinal center survive by upregulating expression of bcl-2. Follicular lymphoma cells which carry the t (14;18) have an internal survival signal and do not require antigenic selection in order to escape apoptosis. Interestingly, cell death is not completely deranged in follicular lymphoma, since Ig heavy chain variable region gene analysis demonstrates persistence of an Ag-driven selection process and presumably therefore death of unselected mutants in follicular lymphoma (71,73). A consequence of bcl-2 overexpression is an increased chance of the t (14;18) positive B cell encountering an appropriate antigen, which will lead to somatic hypermutation of the Ig variable region, expansion,
and survival of the B cell (53, 71). It has been experimentally shown that the Ig molecules on the surface of follicular lymphoma cells undergo somatic hypermutation. However despite the presence of somatic hypermutation, the specificity of the Ig molecule is conserved (73, 74). This shows that antigen selection is important for the maintenance of the lymphoma.

Bcl-2 expression alone is insufficient to induce lymphoma development, as evidenced by the fact that although the t (14;18) is an early event occurring at the pre B-cell stage in the bone marrow (75), cancers associated with the t (14, 18) are peripheral lymphomas of mature B cells (76). As well, although bcl-2 transgenic mice develop polyclonal follicular hyperplasia, only some (11%) develop high-grade lymphomas after a long latency period (77). Also many normal individuals have the t (14:18) in tonsils and peripheral blood lymphocytes (78). These findings suggest that the t(14;18) occurs as an early event in the multi-step process of lymphomagenesis, leading to prolonged survival of the cell, which predisposes it to further transforming genetic mutations. Other oncogenic events that have been shown to co-operate with bcl-2 include myc upregulation (79), p53 inactivation and deletions of chromosome 6 at 6q27 (80). Over time a significant fraction of follicular lymphomas evolve into an aggressive lymphoma with a diffuse large cell architecture, and this is sometimes accompanied by p53 mutation and deletion in addition to the pre-existing bcl-2 lesion.

BCL-1 (CCND1) - Other oncogenes that are involved in NHL include BCL-1, also known as cyclin D1 (CCND1). The t(11;14)(q13;q3) translocation, most commonly found in mantle cell lymphoma, results in the juxtaposition of the JH segment of the Ig gene locus to the Bcl-1 oncogene (81). Bcl-1
together with activated cyclin-dependent kinase 4 (CDK4) phosphorylate the retinoblastoma protein, thereby inactivating it, and releasing E2F, a transcription factor responsible for activating a series of genes required for cell division. The oncogenic activity of Bcl-1 overexpression is most apparent in transgenic mice: when overexpression is targeted to mammary epithelial cells, tumor formation eventually results (82). In the case of lymphoid cells, however, the ectopic expression of CCND1 is not transforming, and the oncogenic ability of CCND1 appears to depend on synergy with other oncogenes, specifically myc (83).

D. (ii) TUMOR SUPPRESSOR GENES

The function of TSGs is to inhibit cell growth. Since a single copy of the gene is sufficient to exert its physiologic role, inactivation of TSGs in human tumors occurs biallelically, most commonly through deletion of one allele and an inactivating mutation of the other. The only TSG known to be involved in NHL is p53. However, it has been suggested that the regions of 6q and 13q32 may contain TSG, as deletions of these regions have been reported in NHL, either in association with other cytogenetic abnormalities or alone (80, 84).

D. (iii) VIRAL INFECTION

Finally, there is a role for viral infection in NHL. The structure of the Epstein Barr Virus (EBV) in some respects resembles a human chromosome in that it contains unique DNA sequence elements (presumably genes) interspersed with repetitive sequence elements. Unlike most other DNA tumor viruses its genome rarely integrates into the host chromosome. The repeat copies differ from individual to individual, and most of these sequences are transcribed and translated (339).
The EBV has been identified in 100% of the cases of endemic Burkitt's lymphoma (BL) from Africa. 75-50% of Latin American BL, but no more than 25% of sporadic BL (340). It has also been identified in 30% of acquired immunodeficiency syndrome (AIDS) Burkitt's lymphoma (341). The EBV is able to alter the growth of B cells significantly (86). This property has allowed the EBV, along with mutant p53, to be used as a means of immortalizing cells in culture. Furthermore, EBV-infected lymphomas usually display a single form of fused EBV termini, indicating that the lymphoma cell population represents the clonally expanded progeny of a single infected cell (85).

Taken together these findings point to a pathogenetic role of EBV in NHL. One model for the development of BL suggests that EBV latent gene expression may drive early polyclonal B-cell proliferation, allowing for other genetic alterations (i.e. c-myc deregulation, and p53 disruption). The fact that c-myc deregulation. and p53 disruption, occur in 100% and 30-40% of BL cases respectively, lends credence to this theory (341). It has even been suggested that the latent proteins of EBV may interact with the p53 protein to lead to overexpression and loss of function of the p53 protein in some forms of lymphoma (342, 343), although this is a controversial issue (344).
II. THE P53 PROTEIN

A. GENE AND PROTEIN STRUCTURE

The human p53 gene contains eleven exons and consists of 20 kb of genomic DNA (122). It is located on a single locus on chromosome 17p13 (123). Exons 2 to 11 are coding sequences with exon 1 separated from exon 2 by a 10 kb intron (122). The minimum p53 promoter required for basal p53 promoter activity is an 85 base pair region upstream of exon 1 (124). The p53 mRNA is 2.6 kb with a large 3' untranslated region (125). The 3' UTR has been implicated in the regulation of p53 mRNA translation (126, 127). A U-rich sequence followed by an Alu-like element on a 330 nucleotide region at the distal end of the human p53 3'UTR represses translation of mRNA in vivo and in vitro and was thus identified as a negative regulatory element. Interaction of this region with repressor proteins may prevent initiation of translation. The 3' UTR may participate in translational regulation of p53 following irradiation (126).

P53 is a nuclear phosphoprotein and consists of 393 amino acids (128). During the S phase of the cell cycle or in response to DNA damage (129, 130), it accumulates in the nucleus via its nuclear localization sequence (residues 316-325) (131, 132, 133, 201). It can be divided into five functional domains, and contains six serines (134). The N-terminus is a highly charged acidic region encompassing the first 42 amino acids. It contains a transactivation domain which is able to interact with the basal transcription machinery to regulate the expression of p53 target genes as a consequence of the binding of the central DNA-binding domain to DNA in a sequence specific manner (130, 135, 136). This region contains one of the segments of p53 that is highly conserved
Figure 3 – Schematic Representation of The P53 Molecule. The human p53 protein consists of 393 amino acids. Highlighted are the N-terminal acidic region (1-42) (ref. 128) which contains the transactivation domain (ref. 138, 201), and one of the evolutionarily conserved regions (I); the proline-rich region (a.a. 63-93, ref. 143); the sequence-specific DNA binding region (a.a. 100-293) which contains four of the evolutionarily conserved regions (II-V) (ref. 145, 201); and the c-terminal region which contains the nuclear localization domain (a.a. 316-325, ref. 201), the oligomerization domain (ref. 201, 148), and a putative DNA damage recognition domain (a.a. 311-393, ref. 201). The 30 c-terminal amino acids form a basic region that is involved in SS DNA and RNA binding and also in the regulation of specific DNA binding (ref. 154, 155, 156). Amino acid positions that contact zinc in the central DNA binding domain (amino acids Cys-176, His 179, Cys-238, Cys-242) have also been indicated (ref. 201). Vertical lines above the schematic represent missense mutations; lines below the schematic represent non-missense mutations (nonsense, frameshift, splicing, silent)(ref. 338).
among different species (137). Residues 17 to 29 of the transactivation domain form an amphipathic helix which is used in interactions with the basal transcription apparatus, as well as binding with the regulatory protein MDM2 (138). The hydrophobic face of the amphipathic helix is used for these interactions (138). The p53 amino acids F19, L22, and W23 are required for transcriptional activation by the protein in vivo (139, 142). Murine double minute 2 (MDM2) gene binds to specific residues at the N-terminus (particularly residues F19, W23, and L26), which are also involved in transactivation and thus inactivates p53 by concealing its transactivation domain (138). The MDM2 protein also accelerates degradation of p53 protein, possibly through the ubiquitin pathway (140, 141). To bind to DNA and activate transcription, p53 must be located in the nucleus. Part of the influence of MDM2 on p53 proteolysis is its ability to enhance transport of p53 protein out of the nucleus and into the cytoplasm where it is targeted for degradation (140, 142). The p53 protein upregulates expression of the MDM2 gene (130), indicating that the MDM2 protein is involved in a negative feedback loop for the p53 protein. The region between the N-terminal activation domain and the central DNA-binding domain (amino acids 63-93) contains five repeats of the motif proline-XX-proline and is capable of binding to SH3 domains (143). This region has been implicated in p53-mediated cell cycle arrest and apoptosis (143, 144).

The central DNA-binding domain of p53 confers the ability of p53 to act as a transcription factor, and is localized between amino acid residues 100 and 293 (145, 201). The core domain structure consist of a B sandwich that serves as a scaffold for two large loops and a loop-helix motif. The two loops, which are held together in part by a tetrahedrally coordinated zinc atom, and the loop-sheet helix motif form the DNA binding surface of p53. Recognition relies on both the major
and minor grooves of the DNA. There are a number of critical residues that contact DNA and others essential for the stability of the amino acid backbone that orient those contact residues. Specifically residues K120, S241, R273, A276, and R283 make contacts with the phosphate backbone in the major groove, while K120, C277, and R280 interact via hydrogen bonds to the DNA bases. Residue 248 interacts via hydrogen bonds to the DNA bases in the minor groove (145).

This region contains four evolutionarily conserved regions where more than 90% of missense mutations are found. The residues most frequently mutated in cancer are at or near the protein-DNA interface. The hot spots are Arg 248 with 9.6% of the mutations, Arg 273 with 8.8%, Arg 175 with 6.1 percent. Glycine 245 with 6.0 %, Arg 249 with 5.6%, and Arg 282 with 4%. Arginine 248 makes the minor groove contact, and Arginine 273 contacts a backbone phosphate. The other four hotspot residues play a critical role in stabilizing the structure of the DNA binding surface of p53 (145). All these mutations block p53 DNA binding and transactivating activity and have been termed “loss of function” mutations (146).

The p53 protein is a tetrameric transcription factor and oligomerization is essential for the tumor suppressor activity of p53 (128, 147, 148). Oligomerization deficient p53 mutants cannot suppress the growth of carcinoma cell lines (147, 148). If one p53 allele is mutated, the mutant protein can exert a “dominant negative” effect on the wild type by participating in multimeric complexes (149, 150, 151). The oligomerization domain encompasses the residues between 316 and 360 (148, 201). The monomer consists of a B strand (326-333) and an α-helix (335-354) (148). The transition from the B strand to the helix occurs over glycine 334. One monomer associates with a
second monomer to form a dimer and two of these dimers associate across a parallel helix-helix interface to form the tetramer. A flexible linker of 37 residues (287-323) links the sequence-specific DNA binding domain to the tetramerization domain (148). As a tetramer, p53 binds specifically to a sequence consisting of two copies of the 10 bp motif PuPuPuC (A/T)(A/T)GPyPyPy3' which can be separated by 0-13 bp (152, 153).

The C-terminal fragment (residues 311-393) can recognize primary DNA damage in the form of insertion/deletion mismatches and binds single-stranded DNA ends (154, 155, 209). The C-terminal 30 amino acids form an open protease sensitive domain which is rich in basic residues (154). It regulates the specific DNA binding by the core domain, turning the latent inactive p53 into the active form (156). Deletion of this domain, phosphorylation at residues S378 or S392 (157), as well as short single strands (20-39 nucleotides) of DNA interacting with this C-terminal domain all activate site-specific DNA binding by the central domain (158).

B. BIOLOGIC AND BIOCHEMICAL PROPERTIES

The p53 protein has a rapid turnover with a short half-life (about 20 minutes) (132). In unstressed cells p53 is present at low levels, and in a latent inactive form (161). Translational (126, 127), transcriptional (159), and post-transcriptional regulation (160) have been evoked in p53 regulation, although, for the most part p53 regulation is achieved by post-translational mechanisms.

Certain kinds of cellular stress including DNA damage (e.g. double-strand breaks in DNA produced by γ-irradiation, or chemical damage to DNA) (132, 133, 162), ribonucleotide triphosphate depletion (163), hypoxia (164), and oncogenic stimuli (165, 166) cause an increase in the level and
activity of the p53 protein. Stabilization of the p53 protein with its subsequent accumulation and its activation from a latent form are regulated independently by different phosphorylation events (167). Hypoxia increases p53 protein levels, partly due to induction of hypoxia-inducible factor α (HIF-α) (168), which can bind to, and stabilize p53. Regions of hypoxia are common features of tumors, as they outgrow their blood supply. That hypoxia upregulates the expression of p53, thus leading to cell cycle arrest and apoptosis of cancer cells, represents yet another way that p53 guards against the formation of cancers (169).

P53 has six serines which can be phosphorylated and can in this way regulate the biochemical functions of the protein (170). Several kinases have been implicated in the phosphorylation of p53 in vitro. These include casein kinase 1 (serine 6 and 9) (171), ataxia-telangiectasia (ATM) (serine 15) (174, 175), cyclin-dependent kinase-activating kinase (CAK) (serine 33) (176), cyclin-dependent kinase-2 (CDK2) and CDC2 (serine 315) (177, 178), protein kinase C (serine 378) (179, 180) and casein kinase II (serine 392) (180, 181). The ATM protein contains a protein kinase domain (173, 174) and cells defective in the ATM gene have a delayed and attenuated p53 response to ionizing radiation (182). The p53 protein is inert as a transcriptional activator in cells lacking detectable DNA-PK activity, suggesting that DNA-PK is upstream of p53 and is required for its transcriptional function (183).

The N-terminal serines are involved in the transactivation function of the protein (184, 185, 186). Modification of the serine 15 site by phosphorylation is important for altering p53 after DNA
damage (137). The serine 15 phosphorylation site is juxtaposed to the MDM-2 binding site and phosphorylation of p53 at this site can block its interaction with MDM-2 and thus lead to stabilization of the protein (172). The c-terminal phosphorylation sites play a role in the DNA binding activity of the protein. Phosphorylation at residue 378 by protein kinase C or residue S392 by casein kinase II activates site-specific DNA binding by the central domain (157). Acetylation of p53 at the c-terminal lysine residues also occurs in response to DNA damage in cells and can also enhance sequence specific binding of p53 (187, 188).

Different physiologic stimuli lead to phosphorylation of different sites. For instance ionizing radiation (IR) results in de novo phosphorylation of serine 15 (172, 189), whereas ultraviolet (UV) but not IR induces phosphorylation of murine serine 389 (190, 191). Different mechanisms are used by the cell to alter the interaction between MDM2 and p53 after DNA damage and oncogene activation (151, 172, 192, 193, 194). A number of studies have looked at how viral and cellular oncogenes stabilize p53 (195, 137). The product of the alternate reading frame (ARF) located within the p16 Ink4a locus (murine p19 ARF, human p14 ARF) can bind to MDM-2 (194, 196, 197, 198) and prevent its destruction of p53 (195). Overexpression of p19 in wild-type but not p53 -/- cells causes cell cycle arrest, suggesting that ARF acts upstream of p53 (194). The E1a DNA tumor virus product as well as the myc oncogenes have both been shown to induce ARF (192, 193, 198).

The p53 protein orchestrates a number of cellular responses. These include cell cycle arrest, apoptosis, induction of repair and direct inhibition of DNA replication (128). The function of the p53 tumor suppressor protein is required for arrest of cell cycle progression in G1 phase following
DNA damage (133, 196). Cells with an intact p53 pathway exhibit γ-ray induced G1 arrest, but cells with p53 gene mutations, or cells expressing viral oncogenes, whose products bind to and inactivate p53, lack a G1 arrest response to DNA damage (133, 199, 200). Delays at the G1/S check point permit repair of damaged DNA prior to DNA replication, thus minimizing the propagation of genetic errors and increasing the chances of cell survival. While cell cycle arrest allows time for DNA repair prior to DNA replication and mitosis, apoptosis rids the organism of cells with severely damaged DNA (201). The p53 protein up-regulates expression of the human bax gene which is to a large extent responsible for p53-dependent apoptosis (203-205).

P53 is involved in the regulation of DNA repair. It was found to interact with, and induce GADD45 (growth arrest and DNA damage-inducible gene) (182), which encodes a protein involved in DNA repair (205-208). P53 also binds single-stranded DNA and catalyzes DNA renaturation and DNA strand transfer, and thus may play a direct role in the repair of damaged DNA (209, 210). As well p53 binds to several helicases. These include the helicases ERCC2 and ERCC3 which make up the subunits of the basal transcription-repair complex TFIH (209-212). P53 binding to these helicases can block their activity in vitro. It is postulated that this may provide yet another mechanism by which p53 is involved in DNA repair and replication (211, 212).

Several studies have shown that p53 plays a direct role in senescence (213). P53-deficient murine cells from a p53 nullizygous mouse readily escape senescence and produce aneuploid immortalized cell lines (214). This is most likely due to the loss of p53-mediated control over centrosome duplication (215) and the G2/M checkpoint preventing re-initiation of S-phase prior to
mitosis or the next G1 Phase (214). As well, when p53 is mutated it can significantly prolong the life span of human diploid cells (213).

P53 may play a role in normal development. Although p53-null mice develop fully and produce fertile gametes (216), some p53-/- mice exhibit a range of developmental abnormalities that are associated with defects in neural tube closure (217, 218). This is the only site of p53 expression in the unstressed, developing mouse (219). P53 null mice have a high incidence of tumor development, developing spontaneous lymphomas and sarcoma in the first six months of life (216). This suggests that the protein plays an important part in the prevention of tumourigenesis. In addition, p53 may be involved in the process of differentiation in hematopoietic cells (220, 221). When p53 is constitutively expressed in hematopoietic cells it induces either differentiation or apoptosis and may therefore be involved in the differentiation pathway of these cells (221, 222).

C. CELL CYCLE ARREST

A cell can incur DNA damage due to a variety of agents in the environment, eg. irradiation, chemical agents (201). One response to this damage is cell cycle arrest, which has the effect of allowing repair of damaged DNA. The cell can arrest before DNA synthesis (G1:S arrest) or before segregation of chromosomes (G2 arrest) (159, 223). P53 plays a major role in G1 arrest after DNA damage (133, 172, 183). Cells expressing mutant p53 are unable to induce this arrest and are therefore genetically unstable (133, 199, 200, 224).

Overall there is a good correlation between sequence-specific transactivation by p53 and the induction of cell growth arrest (225, 226). However, it has been shown that p53 can also inhibit cell
growth in the absence of sequence-specific transactivation (227, 228).

Transactivation of the p21 gene is to a large extent responsible for p53-induced cell cycle arrest (229). The p21 protein, which belongs to the CipKip family of cyclin-CDK inhibitors, causes G1 cell cycle arrest by binding and blocking the function of cyclin D-CDK4/6 complexes (229, 230, 236). Cyclin D-CDK 4 complexes normally phosphorylate the retinoblastoma protein (201, 209). Hypophosphorylated Rb remains associated with E2F, preventing transcription of genes required for S phase progression (230, 232). E2F is a transcription factor which regulates the expression of a number of genes (including those encoding cyclin E, cyclin A, and proliferating cell nuclear antigen (PCNA) required to initiate and to permit progress within the S phase of the cell cycle (233, 234, 235). The retinoblastoma protein, however, is not necessary for the p53/p21 induced G1 arrest since overexpression of either p53 or p21 in Rb defective cells still induces G1 arrest (230,201). P21 binds to PCNA and the evidence suggests that p21-PCNA complexes block the role of PCNA as a DNA polymerase processivity factor in DNA replication but not its role in DNA repair (244, 237). However there is a p21-independent pathway that contributes to the p53-mediated G1 arrest, because cells derived from mice deficient in the p21 gene are only partially deficient in their ability to arrest cells in G1 in response to DNA damage (238). The p53 protein upregulates expression of the GADD45 (growth-arrest and DNA damage inducible)gene (182), which can contribute to this p21-independent cell cycle arrest. The GADD45 protein is involved in the repair of damaged DNA (239). It also binds PCNA (240) and interacts with p21 (244). In addition, it plays a role in DNA damage activated growth arrest. GADD 45 and p21 share an overlapping binding site on the PCNA molecule (241) and their interaction with the protein is likely involved in the regulation of the cell
cycle. Another p53 target gene that may function in this pathway is the insulin-like growth factor binding protein that binds IGF and thus inhibits IGF-mediated growth signaling (242). As well p53 can act to repress transcription of PCNA (244).

P53 has also been implicated in the G2/M phase checkpoint (201). Over-expression of p53 can inhibit entry into mitosis (243, 245). When mitotic spindle inhibitors, such as nocodazole are added to cells with wild type p53, the cells are blocked in G2 (214). In the absence of wild-type p53, these cells will reinitiate DNA synthesis, increasing the ploidy of the cells (214,215). The p53 protein is also involved in centrosome homeostasis. Embryo fibroblasts from p53-null mice acquire more than two centrosomes, leading to mitosis with more than two spindle poles and mitotic failure (215).

D. APOPTOSIS

Programmed cell death occurs through characteristic morphologic changes that include cell shrinkage, nuclear condensation, DNA fragmentation, and plasma membrane blebbing (246). These changes result from the activity of caspases which are intracellular cysteine proteases (247). P53 mediated apoptosis depends on both a p53-mediated transcriptional activity and a p53 activity not requiring transcription. In several cell types, p53-mediated apoptosis initiated by DNA damage occurs in the presence of actinomycin-D, which blocks RNA synthesis (248). Residues 1-214 of the p53 protein fail to bind to DNA or act as a transcription factor, yet the introduction of a p53 cDNA fragment that encodes amino acid residues 1-214 into HeLa cells induces apoptosis (249). Similarly, p53 protein with mutations in the transactivating domain which are unable to activate transcription
can still induce apoptosis in HeLa cells (249). However, p53 proteins containing mutations that alter p53-specific DNA binding or transcriptional activation do not induce apoptosis in baby rat kidney cells (250). Therefore, p53 may use transcriptional activation or direct protein signaling or both to initiate apoptosis, and the choice likely depends on the cell type or experimental situation.

The p53 protein transcriptionally regulates the activity of several genes that are implicated in the apoptotic response. These include BAX (204), IGF-BP3 (242), and Bcl-2 (244). The Bax gene which encodes a product that counters the ability of Bcl-2 to protect cells against apoptosis (251), is transcriptionally activated by wild type P53 (203, 204). Bax may cause cell death by forming a pore (252) in mitochondria that causes release of cytochrome C into the cytosol (253). Cytosolic cytochrome c activates caspases, which then kill the cell (254). Bax is upregulated only in those wild type p53 lines that commit to apoptosis following p53 activation (255). As well, in those cells that commit to apoptosis, Bcl-2 mRNA levels decline following p53 activation due to repression of the Bcl-2 gene by p53 (201, 202). Therefore p53 dependent apoptosis might arise as the balance between BAX and Bcl-2 is tipped towards Bax. However, Bax is not essential for p53-induced apoptosis, since DNA damage induces p53-dependent apoptosis in Bax-deficient thymocytes (256).

Transactivation of the IGF-BP3 gene may also play a role in p53 induced apoptosis by blocking an IGF-mediated survival signal (242, 257). A novel p53-induced gene PAG 608, has also been identified which can cause cell death by an unknown mechanism when transfected into cells (258). Finally a group of p53 inducible genes which increase cellular oxidation has been identified.
P53-mediated apoptosis was inhibited when oxidation was blocked, implying that cellular oxidation is involved in p53 mediated apoptosis (259).

E. CELL CYCLE ARREST OR APOPTOSIS

Cell cycle arrest and apoptosis are two different functions of the p53 protein. They can be separated through the action of cytokines (260), and by generating mutants that are deficient in one but not the other function (261). The choice of a cell to enter the cell cycle arrest pathway or the apoptotic pathway in response to p53 is dictated by multiple factors (262), including the presence of growth and survival factors (263, 264), the presence of p21 protein (265), and the expression of the Rb protein (266). P53 mediated apoptosis can be suppressed by growth factors, which in turn produce a more stable G1 arrest (263). Deletion of p21 can cause a cell that would otherwise undergo p53-dependent cell cycle arrest to undergo apoptosis instead (265). Furthermore, the inactivation of Rb results in the loss of G1 arrest and induction of apoptosis after DNA damage (266). This might be due to the release of E2F, which when over-expressed on its own can induce apoptosis (267). Furthermore, over-expression of Rb blocks p53-mediated apoptosis (268). Other factors such as the extent of DNA damage and the levels of p53 also affect the choice between cell cycle arrest and apoptosis. Using a tetracycline-regulated p53 gene, it was shown that low or moderate levels of p53 result in cell cycle arrest, but high levels trigger apoptosis (269).

F. LI-FRAUMENI SYNDROME

Li-Fraumeni syndrome is a rare (0.01% in the general population) autosomal dominant
inherited syndrome consisting of the following clinical characteristics: a proband with either acute lymphocytic leukemia, sarcoma, breast cancer, and/or adrenocortical carcinoma before the age of 45; a first degree relative with a cancer in this age group, and a first or second-degree relative with sarcoma at any age or any cancer before age 45 (270). Individuals with this syndrome have a germline mutation of p53, which provides the first hit for carcinogenesis. The developing cancers have loss of the wild-type allele (second hit) and retain the mutant p53 allele (270). The tumors associated with LFS are similar to those associated with p53 mutations. Soft-tissue sarcomas usually develop in the first 5 years of life. Acute leukemia and brain tumors occur throughout childhood and young adulthood. In young adults, breast cancer is the most common neoplasm. In addition, there is a high frequency of second malignancies, with almost 50% of affected family members developing more than one neoplasm (271).

G. P53 IN CANCERS

The p53 tumor suppressor gene is one of the most commonly altered genes found in human cancer (up to 50% of cases) (272, 273). P53 mutations have been documented in different types of myeloid leukemia (274, 275), lymphoproliferative disorders such as Burkitt’s lymphoma (276), non-Hodgkin’s lymphoma (277-279) and lymphoid leukemia (280, 281), as well as solid tumors of brain, breast, lung, colon, stomach, and skin (272, 282). Mutation of the p53 gene does not cause malignancy, but rather allows for the accumulation of further genetic alterations in the cell which can lead to malignancy.

Wild type p53 function is lost due to a combination of allelic loss, point mutations and deletions involving both alleles (283). The evolutionarily conserved regions II-IV contain more than
90% of these mutations. Most mutations fall within exons 5-8, which contain the sequence-specific binding domain (220). However, most analyses are confined to exons 5-8, since early studies noted that mutations occurred primarily in this region. Therefore a bias against identification of DNA sequence alterations outside this region may be expected.

P53 mutations occur in relatively early, benign lesions for cancers of the skin (284), lung (285), and head and neck (286). In contrast they occur late in the development of tumors of the colon (287), and thyroid (288). Several studies have found an association between poor survival and mutations in the p53 gene in breast cancer (289), lung cancer (290), childhood T-cell acute lymphoblastic leukemia (291), and ovarian cancer (292). Furthermore, numerous studies have found an association between poor survival and accumulation of the p53 protein in breast cancer (293) colorectal cancer (294), gastric cancer, lung cancer, and ovarian cancer (273). One fourth of all p53 mutations in tumors are transitions at CpG dinucleotides, which are likely to have arisen by deamination of 5-methylcytosine, yielding thymine and thus a C to T mutation (272). Mutation patterns vary among cancer types. Guanine to thymine transversions (G:C to T:A) are the most common mutation found in lung cancers (272). Tandem transitions at G:C base pairs (GC to AA or CC to TT) are the distinguishing feature of p53 mutation in squamous and basal cell carcinoma of the skin. In colon cancer G:C to A:T transitions constitute the majority of mutations and most of these occur at CpG dinucleotides (272).

H. P53 MUTATIONS IN NHL

P53 mutations are generally less frequent in hematological malignancies but when present
are important determinants of outcome (295). Several papers have described investigations of p53 mutations in a variety of hematological malignancies, analyzing the entire coding sequence or exons 4-8 and found that all mutations fall within exons 5-8 (296-298). However, in one paper describing the entire coding sequence of 22 high grade NHL patients, 2 out of 10 mutations were in exon 4 (299). As well, some authors have reported a clustering of mutations in exon 6 in Burkitt’s lymphoma (196). In lymphomas and leukemias, transitions at CpG dinucleotides constitute a major fraction of the point mutations. G to T transversions are uncommon and A:T to G:C transitions predominate among substitutions at A:T pairs (300).

In NHL, the incidence of p53 mutations varies according to histologic type and disease state. Aggressive, high-grade B-cell NHL has about a 30% incidence of p53 mutations. Indolent B-cell NHL rarely have alterations of p53 (277-279), but their progression to high-grade lymphoma can be associated with development of p53 mutations. For example, serial biopsies of patients with follicular NHL who underwent histologic transformation showed that one-third of the transformed samples acquired a p53 mutation that was not detected at the follicular stage of the disease (301). In another study, 4 of 5 cases of transformation of follicular to diffuse large-cell NHL were associated with p53 mutations (277). About 10% of T-cell NHL also have p53 mutations. P53 mutations occur in approximately 35%-45% of Burkitt’s lymphomas. A small stretch of 33 amino acids (codons 213 to 248) contains more than 50% of these mutations. Codon 273, which is often mutated in solid tumors, is rarely altered (302).
I. METHODS OF P53 MUTATION DETECTION

Several different strategies have been used to check for mutations in the p53 gene. These can be broadly divided into functional and genetic assays. Functional assays assess the ability of the p53 protein to act as a transcription activator, its ability to produce apoptosis following irradiation, and its growth suppressing activity. In one assay, human p53 mRNA is reverse transcribed and cotransformed into S. Cerevisiae. This leads to constitutive expression of the p53 protein, which then activates the ADE2 gene because its promoter contains the p53 binding site. The ADE2 gene allows the cell to manufacture adenine on medium limiting for adenine. Colonies are grown on medium limiting for adenine. Consequently yeast colonies containing wild-type p53 turn white and colonies containing mutant p53 turn red. Red colonies are smaller than normal because adenine is limiting for growth. This assay tests the critical activity of p53 and can therefore distinguish inactivating mutations from functionally silent mutations. The technique can be used to screen large numbers of samples and multiple clones per sample for biologically important mutations. The benefit of this technique is that mutations can be detected in tumor specimens contaminated with large amounts of normal tissue.

The apoptotic assays rely on the induction of apoptosis following γ-irradiation (304). Several techniques can be used to detect apoptosis including TUNEL (Tdt-mediated dUTP nick end labeling), FACS (fluorescence activated cell sorting), and DNA ladder. These techniques rely on the fact that DNA is cleaved during apoptosis. Peripheral blood lymphocytes of patients with a germline defect in p53 have a reduced apoptotic response following 4 Gy compared to peripheral blood lymphocytes from normal individuals. For normal individuals the difference in the percentage
of apoptotic cells of irradiated and unirradiated samples varies between 37% and 55%. Patients with germline defects in p53 have 1% and 2% apoptotic cells in unirradiated and irradiated cells respectively. The drawback with this assay however is that defects in the pathway upstream or downstream of p53 cannot be ruled out (304). The growth suppressing activity of p53 can also be tested by a functional assay. Transfection of Saos-2 cells with wild-type p53 cDNA vector results in a reduced plating efficiency, indicating that the expression of p53 is suppressing growth. However, mutant p53 vectors have a reduced ability to suppress the growth of Saos-2 cells (305).

Normal p53 has a very short half-life and is undetectable by immunohistochemistry (IHC) and immunocytochemistry (ICC), whereas p53 proteins which carry a mis-sense point mutation have a prolonged half-life and become detectable by these techniques (306). Therefore, immunocytochemistry (ICC, performed on cell aspirates, including blood, or bone marrow smears) and immunohistochemistry (IHC performed on tissue preparations), using monoclonal antibodies (e.g. Pab240) against p53 epitopes are widely used. There is a very good correlation between the presence of a missense mutation by SSCP and a positive ICC in AML, ALL, CLL, and MDS (307). However the specificity of positive IHC findings in lymphoma is less important. Indeed in NHL a variable number of cases with positive cells by IHC (usually those with less than 25%-30% positive cells by IHC) but without evidence of a p53 mutation by SSCP or direct sequencing have been reported (308). This may be due to the accumulation of increased quantities of normal p53 or to the presence of p53 mutation in a small number of cells (which would be undetectable by SSCP or direct sequencing). Also IHC and ICC are unable to detect p53 mutations other than mis-sense mutations (chain termination, codon mutations, and splice site mutations) that do not lead to
increased p53 levels and constitute about 15% of all mutations (295).

For analysis of genes several different approaches can be employed. Southern blot analysis can be used to detect gross abnormalities like large deletions and insertions. The advantage of this technique is that no detailed knowledge of the structure and sequence of a gene is required (309).

An indirect detection method which takes advantage of DNA polymorphism is the analysis of loss of heterozygosity, (LOH) (310). When one p53 allele develops a point mutation, then the probability of loss of the normal p53 allele through recombination is increased (310). Several polymorphic sites that are informative in this regard are present in the region of the p53 gene (311). The drawback of this approach, however, is that LOH can occur in the absence of a detectable p53 mutation (312).

For more subtle abnormalities such as single base pair mutations, single-strand conformation polymorphism can be employed. Single strand conformations are highly sequence specific and distinctive in electrophoretic mobility. These conformation mobility differences are exploited for detection of sequence variants. The PCR-SSCP technique detects an abnormality in less than 10% DNA containing mutant p53 in a background of 90% DNA containing wild type p53 (313, 314). For 100-300 bp fragments, the specificity of PCR-SSCP is more than 95% (315). The most appropriate use for SSCP analysis is when looking for a variety of unknown mutations. It is well suited for screening a large number of samples to detect mutations of various types. Other screening methods which can be employed include denaturing gradient gel electrophoresis, which relies on differential electrophoretic migration of wild-type and mutant double stranded DNA through a gradient of increasing concentrations of a denaturing agent for the detection of mutations (316). Direct sequencing is used for definitive analysis of genetic alterations.
CHAPTER II – METHODS

I. BIOLOGIC PROGNOSTIC FACTOR STUDY

The Biologic Prognostic Factor Study was a large collaborative study organized by investigators at the Sunnybrook Health Sciences Center for newly diagnosed patients with Non-Hodgkin's lymphoma. The main objective of the study was to determine clinical, pathologic, and laboratory indices that were important prognostic factors in NHL. The evaluation included a complete history taking and physical examination. Clinical indices such as sex, age, stage (I-II vs. III-IV as determined by the Ann Arbor Classification System), Eastern Co-operative Oncology Group (ECOG) performance status (0-1 vs. 2-4, as defined in table 3), the presence or absence of extranodal disease, nodal disease, B symptoms (as defined by the Ann Arbor Classification system, see table 2, pg 18), and bulky disease (defined as ≥5cm) were evaluated. Routine laboratory indices that were evaluated included CBC, differential, blood film, serum electrolytes and creatinine, liver function tests, serum LDH, chest roentgenography, bone marrow aspiration and biopsy, and computed tomography of the chest, abdomen, and pelvis. Other experimental laboratory indices such as T-cell vs. B-cell phenotype, cell surface markers, t(14:18) translocation, and Ki-67 proliferative index were also assessed. The criteria of the Working Formulation was used for morphologic classification of patient samples.
TABLE 3- ECOG PERFORMANCE STATUS

<table>
<thead>
<tr>
<th>Grade</th>
<th>ECOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Fully active, able to carry on all pre-disease performance without restriction</td>
</tr>
<tr>
<td>1</td>
<td>Restricted in physically strenuous activity but ambulatory and able to carry out work of a sedentary nature, e.g., light house work, office work</td>
</tr>
<tr>
<td>2</td>
<td>Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of waking hours</td>
</tr>
<tr>
<td>3</td>
<td>Capable of only limited selfcare, confined to bed or chair more than 50% of waking hours</td>
</tr>
<tr>
<td>4</td>
<td>Completely disabled. Cannot carry on any selfcare. Totally confined to bed or chair</td>
</tr>
</tbody>
</table>

(Reference 336)

Initial treatment included a combination of chemotherapy, therapeutic surgery or radiation therapy (curative vs. palliative), depending on the Working Formulation (WF) grading of the NHL. Initial therapy for low grade disease included a watch and wait approach for asymptomatic patients and systemic therapy for symptomatic patients. Systemic therapy comprised chemotherapy or radiotherapy alone as well as chemotherapy and radiotherapy in combination. Initial therapy for intermediate and high grade NHL included chemotherapy plus or minus radiotherapy or surgery.

All patients were re-evaluated upon completion of therapy. Follow-up included history and physical examination, blood cell and differential counts, blood-chemistry tests including LDH, and computed tomography of the chest, abdomen and pelvis. The period of follow-up was not consistent for all patients and ranged from 18 months to 69 months. Complete remission was defined as the disappearance of clinically evident-disease and either resolution of all radiologic evidence of lymphoma, or at least a 50% reduction of disease that remains stable for at least 3 months. Response was defined as incomplete if one of the following occurred: progression on therapy or within one
month of completing therapy, biopsy proven persistent disease, or strong clinical suspicion (persistent B-symptoms or LDH).

This thesis will focus on the presence or absence of p53 mutations. The presence of p53 mutation will be determined with the help of the PCR-SSCP technique. The presence of p53 mutations will then be correlated with clinical indices such as sex, age (mean age, and age > 60 years vs. age ≤ 60 years), stage, ECOG performance status, the presence or absence of extranodal disease, nodal disease, B-symptoms, and bulky disease. As well, the presence of p53 mutations will be correlated with serum LDH, and the Working Formulation categories of low grade (non-aggressive) vs. intermediate and high-grade (aggressive) NHL.

II. FRESH TUMOR SAMPLES

Fresh tumor biopsies were divided into two parts. One part was used by the pathologist for diagnostic purposes. The other part was used by technologists at the Sunnybrook Health Sciences Center to prepare genomic DNA. Single cell suspensions were obtained by passing the lymph node through a steel wire mesh. The GENEPURE nucleic acid purification system, Model 341 (Applied Biosystems, Foster City, CA) was used to extract high molecular weight DNA.

III. DNA ISOLATION FROM CELL LINES

Cells were lysed in 10mM EDTA, pH 8.0, 0.5M NaOAC, 0.5% SDS and 50 mg/ml Proteinase K. They were extracted with phenol chloroform, precipitated, resuspended in 10mM Tris, 1mM EDTA with 30ug DNAse free RNase for 2 hours, re-extracted, precipitated and resuspended
in sterile water.

**IV. POLYMERASE CHAIN REACTION**

Between 0.1 and 0.5 ug of genomic DNA was amplified by PCR to detect exons 5-8 of the p53 gene in 133 patient samples. One patient sample, PF82, had only exon 7 analyzed which showed a mutation. The primers used for the amplification are shown in Figure 2. DNA was amplified in 100ml of PCR buffer (Pharmacia) containing 15mM MgCl₂, 200mmol of each deoxynucleotide (Pharmacia), 100 pmol of each primer and 2 units of Taq polymerase (Pharmacia). The reaction conditions were as follows: 40 cycles of denaturing at 94 C for 30 seconds, annealing at 55 C for 30 seconds, and extension at 72 C for 1 minute. PCR products were electrophoresed to check for proper size.

**V. OVERVIEW OF SSCP METHODOLOGY**

A non-radioisotopic single strand conformation polymorphism analysis with a conventional minislab gel electrophoresis apparatus was used (HELIXX TECHNOLOGIES) (317). This technique comprises amplification of DNA fragments by the PCR technique with specific oligonucleotide primers. The primers are chosen outside the DNA fragment that is to be analyzed. The PCR product is then denatured, and electrophoresed on neutral polyacrylamide gels in a conventional minislab apparatus. The SSCP patterns are detected using silver staining. Several electrophoretic parameters (running temperature, buffer denaturants, DNA concentrations, and polyacrylamide gel concentration) influence the degree of strand separation. Gel concentration can be changed conveniently as the company provides pre-made gels of different concentrations. Gel temperature, the most critical parameter influencing SSCP band resolution and reproducibility can be precisely controlled through the use of a thermostatically controlled circulator which accurately
maintains a predetermined buffer temperature with the gel unit. The thermoflow SSCP electrophoresis system consists of an X cell II mini-cell system adapted to allow temperature regulated electrophoresis. It re-circulates 1 x TBE buffer from the mini-cell through a condenser coil regulated by a temperature controlled water bath (Haake, Karlsruhe, Germany).

VI. SSCP PROTOCOL ADAPTED FOR USE WITH X CELL II APPARATUS (NOVEX)

Between 3ul and 6ul of the 100 PCR reaction was diluted 1:4 with a formamide based SSCP loading buffer in individual 0.2ul micro-amp PCR tubes (318). The denaturing buffer was prepared by combining in a 2:3 ratio. a stock denaturing solution with a stock stop solution. Both stocks were stored at room temperature. The denaturing solution was prepared with 0.1% SDS and 10mM EDTA. The stop solution was prepared with 95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanole FF. Samples were denatured at 95% for 5 minutes before being soaked at 4°C in the PCR machine for 5 minutes prior to loading on acrylamide gel (318).

The mixture of denaturing buffer and PCR product was loaded into the separate wells of Novex pre-cast gels (Novex, La Jolla, California). A Biorad, Model 3000Xi Programmable Power Supply (Richmond, CA) was used to provide DC Power.
VII. PRIMERS USED

P5-5: 5'-TTCCCTTTCTGCAGTACTC3'
P5-3: 5'-GGACCCTGGCAACCAGCCC3'
P6-5: 5'-ATAGCGATGGTCAGCAGCTG3'
P6-3: 5'-AGTTGCAAACCAGACCTCAGY
P7-5: 5'-GTGTTGTCTCCTAGGTTGGC3'
P-3: 5'-AAATCGGTAAGAGGTTGGCC3'
P8-5: 5'-TATCCTGAGTGGTGGTAATC3'
P8-3: 5'-TAACTGCACCTGGTCTCC3'

VIII. STATISTICAL ANALYSIS

All analysis was done using SAS software for statistical analysis. A normal score plot was done which showed that the age of the patients at diagnosis did not follow a normal distribution to the extent which would allow utilization of the student’s t-test. Therefore, the Wilcoxon test for comparing means was utilized for the variable age at diagnosis. The correlation of a p53 mutation with LDH, stage, bulky disease, extranodal disease, and sex was done with the help of the 2 tailed Z test for comparing proportions. The 2 tailed Fisher’s exact test was used for correlation of p53 mutations with ECOG status, B symptoms, nodal disease and pathologic classification (low grade vs. intermediate and high grade) because there were not enough observations to use the Z test for comparing proportions. Of patients with p53 mutations, only 3 had ECOG status≥2, 2 had no nodal disease, 4 had B symptoms, and 4 had low-grade pathology. The Kaplan-Meier estimate of survival was used to measure mean survival for patients with and without p53 mutations.
CHAPTER III – RESULTS

I. POSITIVE AND NEGATIVE CONTROLS

DNA samples from the cell lines LY1 (mutation in the p53 gene at codon 158 and 176 in exon 5), LY7 (mutation at codon 245 in exon 7) and LY8C3 (mutation at codon 282 in exon 8) were used as positive controls. R213, a sample known to carry a base pair change in exon 6 was used as a positive control for exon 6 (301). The cell line LY3 which has a wild-type p53 sequence was used as a negative control for exons 5-8.

<table>
<thead>
<tr>
<th>TABLE 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Positive control (Mutant)</th>
<th>Exon 5</th>
<th>Exon 6</th>
<th>Exon 7</th>
<th>Exon 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY1</td>
<td>R213</td>
<td>LY7</td>
<td>LY8 C3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P53 sequence</th>
<th>a.a 158 arg → hist</th>
<th>Codon 213 CGA→CGG arg→arg</th>
<th>a.a 245 gly → asp</th>
<th>a.a 282 arg → pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.a. 176 cys → glyc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control (Wild Type)</td>
<td>LY3</td>
<td>LY3</td>
<td>LY3</td>
<td>LY3</td>
</tr>
</tbody>
</table>

(reference 278,301)

II. DETERMINING THE OPTIMAL CONDITIONS FOR MUTATION DETECTION

Intronic primers were used to amplify exons 5-8 from samples known to have mutations in these exons. These PCR products were then subjected to SSCP analysis at several different temperatures to determine the optimal conditions. The thermoflow system was used to provide precise temperature control. Two different SSCP conditions were chosen for mutation detection for each exon in order to increase the sensitivity of detection (319). These conditions are listed in Table 5.
TABLE 5-Conditions Chosen For Optimal Detection of P53 Mutation For Each Exon

<table>
<thead>
<tr>
<th>EXON 5</th>
<th>CONDITION 1</th>
<th>CONDITION 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20% gel, 30°C, 1xTBE buffer</td>
<td>20% gel, 26°C, 1xTBE buffer</td>
</tr>
<tr>
<td>EXON 6</td>
<td>20% gel, 20°C, 1xTBE buffer</td>
<td>20% gel, 9°C, 1xTBE buffer</td>
</tr>
<tr>
<td>EXON 7</td>
<td>20% gel, 17°C, 1xTBE buffer</td>
<td>20% gel, 21°C, 1xTBE buffer</td>
</tr>
<tr>
<td>EXON 8</td>
<td>4-20% gel, 8°C, 1xTBE buffer</td>
<td>20% gel, 12°C, 1xTBE buffer</td>
</tr>
</tbody>
</table>

III. SSCP ASSAYS

The SSCP gels that were obtained for the positive and negative controls for conditions 1 and 2 for each exon are shown in Figure 4.

IV. SENSITIVITY ASSAY

Tumor samples from patients may contain a combination of cells with mutated and w.t. p53, and as such the sensitivity of mutation detection is important to test the sensitivity of this SSCP technique. DNA from a positive control (the cell line LY1) and negative control (the cell line LY3) for exon 5 was mixed in five different concentrations (LY1/LY3 - 1:0, 1:2, 1:4, 1:8, 0:1) and PCR amplified in five different tubes. These were run in one of the SSCP conditions chosen for exon 5. This technique could detect a mutation in exon 5 in all four tubes which contained LY1 even when the concentration of LY1/LY3 was 1:8. Figure 5 shows the gel for this sensitivity assay.
**Figure 4-Mutant and W.T. SSCP Patterns For Each Condition For Exons 5-8.** The difference in banding patterns between mutant and w.t. is due to conformational changes caused by sequence alterations. The PCR fragment size is 234 b.p., 215 b.p., 213 b.p., and 197 b.p. for exons 5-8 respectively.
Figure 5 - Sensitivity Assay. DNA from a positive control (LY1) and negative control (LY3) for exon 5 was mixed in five different concentrations (LY1/LY3 = 1:0, 1:2, 1:4, 1:8, 0:1) and PCR amplified in five different tubes. These were run in condition 2 chosen for exon 5.
V. ANALYSIS

In total one hundred and thirty four patients were studied. Twenty eight patients were found to have p53 mutations and 106 patients were found to have wild type p53. As discussed in the methods section the presence of a p53 mutation was correlated with clinical indices such as age, stage, ECOG performance status, the presence or absence of extranodal diseases, nodal disease, B symptoms, and bulky disease. These parameters were defined in the Methods Section.

As mentioned previously twenty eight patients were found to have p53 mutations. Table 6 summarizes the SSCP data for the 28 patients with positive SSCP results. Exon 6 had the highest number of SSCP positive results. Fourteen patients had positive results in exon 6, 9 in both conditions, 3 in condition 1 only, and 2 in condition 2 only. Exon 8 was the least affected with one patients positive in both conditions, and two patients positive in only condition one. Nine patients were positive in exon 5, 6 in both conditions, and 3 in condition 2 only. Five patients were positive in exon 7, 2 in both conditions and 3 in condition 1 only. This could however be misleading as exon 6 is reported to be a common site of a polymorphism in the p53 gene (codon 213-CGA→CGG; argining→argining) (301).

The median age for the 134 patients was 63.2 years (range 21.7 to 82.7 years) (Table 7). Patients with a p53 mutation had a median age of 63.2 years versus 64.0 years for patients with a wild-type p53 and this was not statistically significant (p = 0.43). Of the 134 patients 68 were male and 66 were female. The presence of p53 mutations was not correlated with age (>60 years vs.
≤60 years), sex, ECOG status, stage, presence of nodal disease, bulky disease, B symptoms, extranodal disease, or histological classification (Table 7).

The presence of a p53 mutation was correlated with having an abnormal LDH (p = 0.03) (Table 7). Of the 28 patients with mutant p53, 13(56.5%) had normal LDH values, 10(43.5%) had elevated LDH values, and 5 had missing LDH values. Of the 106 patients with wild type p53, 65(79.3%) had normal LDH values, 17(20.7%) had elevated LDH values and 24 had missing LDH values.

From a pathologic standpoint, of the one hundred and thirty four patients, 40 had low grade disease (WF – A, B, C). 81 had intermediate grade disease (WF – D, E, F, G), and 13 patients had high grade disease (WF – H, I, J). Table 8 summarizes the pathologic data for the patients on the study. The 2-tailed Fisher’s exact test was used for comparing patients with low grade NHL to those with aggressive (intermediate and high grade) NHL. When combining the intermediate and high grade categories of the WF into the general category of aggressive NHL, patients with p53 mutations were more likely to have aggressive disease as opposed to non-aggressive (WF of low grade) disease (p = 0.06, Table 7). Of the 106 patients with wild-type p53, 70(66.0%) had aggressive disease and 36(34.0%) had non-aggressive disease. Of the 28 patients with a mutant p53, 24(85.7%) had aggressive disease and 4(14.3%) had non-aggressive disease. However, in the patients with aggressive disease (intermediate and high grade of WF), having a p53 mutation was not correlated with having high-grade as opposed to intermediate grade pathology (p = 1.00, Table 7).
Follow-up survival data was missing for one patient with a p53 mutation. Of the remaining 133 patients, 82 patients remained alive during the study, and 51 patients died during the study. The period of follow-up was not consistent for those patients that were alive at the end of the study, and ranged from 18 months to 69 months. For those patients that died during the study, the time of death after diagnosis ranged from 1.4 to 45.6 months. Of the 106 patients with wild-type p53, 71 (67.0%) were alive during the study and 34 (33.0%) died during the study. Of the 27 patients with mutant p53, 11 (40.7%) were alive at the end of the study and 16 (59.3%) died during the study.

The Kaplan-Meier estimate of survival was used to estimate the survival time for patients with wild-type and mutant p53. Using this method the mean survival time for patients with a p53 mutation was 2.86 ± 0.44 years as opposed to 4.09 ± 0.23 years for patients with a wild-type p53. This was a statistically significant difference (p = 0.0156) (Table 7).
<table>
<thead>
<tr>
<th></th>
<th>Exon 5</th>
<th>Exon 6</th>
<th>Exon 7</th>
<th>Exon 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>11</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>12</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>13</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>14</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>15</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>16</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>17</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>18</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>19</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>20</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>21</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>22</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>23</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>24</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>25</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>26</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>27</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>28</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>29</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>30</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
TABLE 7-CORRELATION OF P53 MUTATIONS WITH CLINICAL AND PATHOLOGIC DATA

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>TOTAL</th>
<th>PATIENTS WITH P53 MUTATIONS</th>
<th>PATIENTS WITH WILD-TYPE P53</th>
<th>P. VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>134</td>
<td>28 (20.9%)</td>
<td>106 (79.1%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (Years)</td>
<td>63.2</td>
<td>64.0</td>
<td>63.2</td>
<td>0.43</td>
</tr>
<tr>
<td>≤ 60 years B no. (%)</td>
<td>53</td>
<td>11 (39.3%)</td>
<td>42 (39.6%)</td>
<td></td>
</tr>
<tr>
<td>&gt; 60 years B no. (%)</td>
<td>81</td>
<td>17 (60.7%)</td>
<td>64 (60.3%)</td>
<td>0.97</td>
</tr>
<tr>
<td>Sex - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>68</td>
<td>14 (50%)</td>
<td>54 (50.9%)</td>
<td>0.93</td>
</tr>
<tr>
<td>Female</td>
<td>66</td>
<td>14 (50%)</td>
<td>52 (49.1%)</td>
<td></td>
</tr>
<tr>
<td>B Symptoms B no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>107</td>
<td>24 (85.7%)</td>
<td>83 (62.0%)</td>
<td>0.44</td>
</tr>
<tr>
<td>Present</td>
<td>27</td>
<td>4 (14.3%)</td>
<td>23 (21.7%)</td>
<td></td>
</tr>
<tr>
<td>Clinical Stage - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I or II</td>
<td>43</td>
<td>7 (25%)</td>
<td>36 (34.0%)</td>
<td>0.37</td>
</tr>
<tr>
<td>III or IV</td>
<td>91</td>
<td>21 (75%)</td>
<td>70 (66.0%)</td>
<td></td>
</tr>
<tr>
<td>Bulky Disease (≥5cm) - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>72</td>
<td>18 (64.3%)</td>
<td>54 (50.9%)</td>
<td>0.21</td>
</tr>
<tr>
<td>Absent</td>
<td>62</td>
<td>10 (35.7%)</td>
<td>52 (49.1%)</td>
<td></td>
</tr>
<tr>
<td>Extranodal Disease - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>70</td>
<td>18 (64.3%)</td>
<td>52 (49.1%)</td>
<td>0.15</td>
</tr>
<tr>
<td>Absent</td>
<td>64</td>
<td>10 (35.7%)</td>
<td>54 (50.9%)</td>
<td></td>
</tr>
<tr>
<td>Lactate Dehydrogenase Level - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>78</td>
<td>13 (56.5%)</td>
<td>65 (79.3%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Elevated</td>
<td>27</td>
<td>10 (43.5%)</td>
<td>17 (20.7%)</td>
<td></td>
</tr>
<tr>
<td>CHARACTERISTIC</td>
<td>TOTAL</td>
<td>PATIENTS WITH P53 MUTATIONS</td>
<td>PATIENTS WITH WILD-TYPE P53</td>
<td>P-VALUE</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------</td>
<td>-----------------------------</td>
<td>----------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Bone Marrow Involvement - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>45</td>
<td>9 (33.3%)</td>
<td>36 (35.0%)</td>
<td>0.87</td>
</tr>
<tr>
<td>No</td>
<td>85</td>
<td>18 (66.7%)</td>
<td>67 (65.0%)</td>
<td></td>
</tr>
<tr>
<td>Nodal Disease - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>125</td>
<td>26 (92.9%)</td>
<td>99 (93.4%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Absent</td>
<td>9</td>
<td>2 (7.1%)</td>
<td>7 (6.6%)</td>
<td></td>
</tr>
<tr>
<td>ECOG Performance Status - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>121</td>
<td>25 (89.3%)</td>
<td>96 (91.0%)</td>
<td></td>
</tr>
<tr>
<td>2-4</td>
<td>13</td>
<td>3 (10.7%)</td>
<td>10 (9.4%)</td>
<td>0.73</td>
</tr>
<tr>
<td>Histologic Type - All Patients - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (Non-Aggressive)</td>
<td>40</td>
<td>4 (14.3%)</td>
<td>36 (34.0%)</td>
<td>0.06</td>
</tr>
<tr>
<td>Intermediate and High (Aggressive)</td>
<td>94</td>
<td>24 (85.7%)</td>
<td>70 (66.0%)</td>
<td></td>
</tr>
<tr>
<td>Histologic Type (Aggressive) - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>81</td>
<td>21 (87.5%)</td>
<td>60 (85.7%)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>13</td>
<td>3 (12.5%)</td>
<td>10 (14.3%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Overall Survival (years)</td>
<td></td>
<td>2.86 ± 0.44 yrs</td>
<td>4.09 ± 0.23 yrs</td>
<td>0.016</td>
</tr>
</tbody>
</table>
### TABLE 8-PATHOLOGIC CLASSIFICATION OF PATIENTS WITH AND WITHOUT P53 MUTATIONS

<table>
<thead>
<tr>
<th>WORKING FORMULATION</th>
<th>TOTAL</th>
<th>PATIENTS WITH P53 MUTATIONS</th>
<th>PATIENTS WITH WILD-TYPE P53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Grade</td>
<td>40</td>
<td>4</td>
<td>36</td>
</tr>
<tr>
<td>A</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>B</td>
<td>14</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>C</td>
<td>19</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>Intermediate Grade</td>
<td>81</td>
<td>21</td>
<td>60</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>E</td>
<td>8</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>F</td>
<td>20</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>G</td>
<td>43</td>
<td>14</td>
<td>29</td>
</tr>
<tr>
<td>High Grade</td>
<td>13</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>H</td>
<td>8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>J</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>
CHAPTER IV – DISCUSSION

In this thesis, I sought to examine the relationship between having a mutated p53 allele with clinical (age, stage, ECOG status, abnormal LDH, presence of B symptoms, bulky disease, nodal disease, extra-nodal disease, and overall survival), demographic (sex), and pathologic (WF grading of low, intermediate, and high) data. The method of SSCP was used for mutation detection. This method can detect a mutation when only one allele is affected, an important consideration because the p53 protein functions in the cell in a tetrameric configuration (320, 149), and some papers have suggested that if one allele is mutated, the mutant protein can exert a “dominant negative” effect on the wild type by participating in these multimeric complexes (149, 150, 151). However, there is likely a partial “dominant negative” effect of the w.t. protein as there is still selection for loss of the other allele in the majority of tumors. The SSCP was done on pre-cast non-denaturing polyacrylamide gels, using a thermoflow SSCP electrophoresis system for temperature control. This method was shown to be sensitive for mutation detection when the ratio of a mutated to w.t. p53 was 1:8. This is critical when dealing with tumor samples from patients which may contain a combination of cells with mutated and w.t. p53.

In total 134 patients (WF – low grade - 40, WF intermediate grade 81, WF-high grade 13) were screened for p53 mutation by this method. Twenty-eight (20.9%) patients were found to have p53 mutations (WF – low grade-4, WF-intermediate grade-21, WF-high grade 3). The proportion of patients with p53 mutations was higher for those who had aggressive disease (intermediate and high grade of the WF – 25.5%) as opposed to those who had low grade disease (10.0%) (p = 0.06). The fact that mutations in the p53 gene are more prevalent in the more aggressive lymphomas is not
surprising since a mutation in the p53 gene will allow the accumulation of further genetic alterations in a tumor cell thereby leading to a more aggressive phenotype. The end result would be a higher proportion of p53 mutations in the aggressive NHLs.

Similar results have been reported by several other groups (321, 301, 322) which have found that p53 mutations are more prevalent in aggressive (intermediate and high grade) NHL as opposed to those with non-aggressive (low-grade) pathology. In the literature, the percentage of p53 mutations in patients with low-grade NHL ranges from 4.2%-13.3% (321, 301, 322). The percentage of p53 mutations in patients with intermediate and high grade NHL ranges from 16.8%-26.5% (321, 301, 322, 323) (Table 9).

One group studied serial biopsies from patients with follicular lymphomas that had undergone transformation to high-grade NHL and found that these transformations were associated with mutations of the p53 gene in 25%-30% of the cases (301). They concluded that p53 mutation was probably acquired during the histologically indolent phase of follicular lymphoma, because rare p53 positive cells (as determined by immunoperoxidase staining) could be found in the majority of the pretransformation biopsies that progressed along the p53 dependent pathway (301). These results support the hypothesis that a p53 mutation is probably not the transforming event, but rather provides the opportunity for other genetic alterations to accumulate leading to the aggressive phenotype of NHL. The fact that p53 is involved in cell cycle arrest and apoptosis after a cell has incurred DNA damage lends further credence to this hypothesis, since the inability to do this would allow the accumulation of genetic alterations.
TABLE 9  PREVALENCE OF P53 MUTATIONS IN AGGRESSIVE VS. LOW GRADE NHL IN OTHER STUDIES

<table>
<thead>
<tr>
<th>Author (Reference #)</th>
<th># of Patients with Low Grade Lymphoma</th>
<th># (%) with P53 Mutations Detected by SSCP and Sequencing</th>
<th># of Patients with Aggressive (Intermediate and High Grade) Lymphoma</th>
<th># (%) with P53 Mutations Detected by SSCP and Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sander CA (#301)</td>
<td>25</td>
<td>3 (12%)</td>
<td>34</td>
<td>9 (26.5) *</td>
</tr>
<tr>
<td>Koduru PR (#321)</td>
<td>118</td>
<td>5 (4.2%)</td>
<td>119</td>
<td>20 (16.8%)</td>
</tr>
<tr>
<td>Wilson W (#322)</td>
<td>15</td>
<td>2 (13.3%)</td>
<td>60</td>
<td>14 (23.3%)</td>
</tr>
<tr>
<td>Ichikawa Â (#323)</td>
<td>-</td>
<td>-</td>
<td>102</td>
<td>22 (22%)</td>
</tr>
</tbody>
</table>

*Two mutations consisted of a common polymorphism in exon 6 (codon 213 CGA→CGG; Arg→Arg)
We found no statistically significant difference in the proportion of patients with p53 mutations between those patients with intermediate pathology (25.9%) and those with high-grade pathology (23.1%) (p = 1.00). One hypothesis that can be put forward is that a p53 mutation probably provides the opportunity for further genetic alterations to accumulate, leading to a more aggressive phenotype of NHL. However, the type and number of these other genetic alterations may differentiate between an intermediate grade versus a high grade NHL.

Having a p53 mutation was not statistically correlated with having bulky disease, B symptoms, bone marrow involvement, nodal disease, a high ECOG performance status (2-4 vs 0-1), a higher stage (3-4 vs 1-2), or extranodal disease.

We found that having a p53 mutation was significantly correlated with elevated lactate dehydrogenase levels (p = 0.03). An elevated LDH level is an indicator of high tumor burden. The p53 protein allows the cell to undergo cell cycle arrest or apoptosis after it has incurred DNA damage. An inability to do this will allow a tumor cell to accumulate further genetic alterations which will then result in uncontrolled tumor growth and proliferation. This will then manifest itself as an increase in tumor burden with a resultant increase in LDH. Elevated LDH levels, along with advanced clinical stage, the presence of >1 extranodal site of disease, elevated ECOG status, and advanced age (>60 years) has been shown to be a predictor of poor outcome in the International Prognostic Factor Study. However, although more patients with a p53 mutation appeared to have extranodal disease, a high ECOG value, a higher stage, and be older than 60 years of age, these differences were not statistically significant (p=0.15, p=0.73, p=0.37, and p=0.97 respectively).
We found that having a p53 mutation was significantly correlated with a shorter overall survival (4.09 ± 0.4 years for patients without a p53 mutation and 2.86 ± 0.2 years for those with a p53 mutation as determined by the Kaplan Meier estimate, p=0.016). Tumor growth is the result of cell proliferation and cell loss. Apoptosis is the most significant component of cell loss in most tumors. (324, 325). In follicular lymphoma and chronic lymphocytic leukemia the primary pathogenic event is the disruption of apoptotic cell death rather than uncontrolled proliferation, which thereby leads to the aberrant accumulation of cells (326). The results described in this thesis are in keeping with this notion, since p53 is essential for cell cycle arrest and apoptosis in a cell after it has had DNA damage. This would allow the cell to either repair the DNA damage or undergo apoptosis if the damage is too severe. Inability to do this as a result of mutation would allow the DNA damage to be propagated to future daughter cells and in turn would create a more aggressive clone. This alone would account for the decreased overall survival observed in patients with a p53 mutation. However there is another contributing factor to the decreased survival. It is known that chemotherapeutic drugs exert their effectiveness by inducing apoptosis in rapidly dividing cells. Some chemotherapeutic agents and radiation induce apoptosis in cells by a mechanism requiring the p53 protein (327, 328, 329). Thus, if patients have a mutation in the p53 gene, it is more likely that they will be resistant to chemotherapeutic agents and radiation. In fact there is a strong correlation between the p53 status of a tumor and the response of the tumor to treatment in animal models (328). A mutant p53 can transactivate the multi-drug resistance gene 1 (MDR1) (330), and as such it has been suggested that a mutant p53 may be an important cause of drug resistance in relapsed or refractory non-Hodgkin's lymphoma (327). Drug resistance would then lead to a decrease in overall survival. These observations are consistent with the results of this thesis which shows that having
a p53 mutation correlates with shorter overall survival.

A strength of the present work is that it is a prospective study, analyzing a large number of patients at diagnosis before the contributing effects of various anticancer treatments. In addition the Working Formulation categories of low-grade, intermediate-grade, and high-grade were all present in the patient population. A weakness of the present work is that the patients did not all receive the exact same treatment, although the principles of treatment were consistent for various clinical presentations.

Several studies have looked at p53 mutations in patients with NHL, and their relation to survival. In one study, 102 previously untreated cases of intermediate and high grade B-cell lymphoma were assessed for mutations in the p53 gene by SSCP and sequencing (323). Patients with tumors harboring p53 mutations were older (mean age of 65 years vs. mean age of 53 years for patients with w.t. p53) (p = 0.001), had a more advanced clinical stage (p = 0.04), and had higher lactate dehydrogenase levels (p = 0.01) than patients without a p53 mutation. Twenty two cases (22%) had p53 mutations, with the rate of complete remission being significantly lower in patients having a p53 mutation (27%) compared to those with the wild-type p53 gene (76%) (p < 0.001). As well, the overall five year survival was significantly lower in patients with p53 mutations (16% vs. 64% respectively, p < 0.001). Multivariate analysis incorporating prognostic factors from the International Prognostic Index demonstrated that p53 mutations had independent effects on the rates of complete remission and survival (323). Most of the results of this paper corroborate the findings of this thesis. However I did not find age, or stage to be statistically correlated with having a p53
mutation. One reason for this discrepancy could be that this thesis examined patients with NHL that had low, intermediate, and high grade disease, as opposed to only patients with aggressive (intermediate and high grade) disease. Low grade lymphomas usually occur in older adults and present with disseminated disease (21). Low grade lymphomas made up 30% of the patient population in this thesis. This would likely affect the correlation of a p53 mutation with age and stage.

Another group analyzed tumor tissue from 75 patients with relapsed/refractory NHL within six weeks of treatment with EPOCH (etoposide, vincristine, cyclophosphamide, and doxorubicin) for p53 expression, and also for p53 mutation in 56 of these patients (322). These findings were correlated with multiple clinical characteristics (including age, LDH level, ECOG performance status, stage, extranodal disease sites, and morphologic subtype), as well as drug resistance and median progression-free and overall survival. Sixteen tumors (21%) had a p53 abnormality. Thirteen tumors had p53 expression with eleven of these having mutations confirmed by sequence analysis. As well, in the tumors without p53 overexpression three showed mutations in the p53 gene. A multivariate analysis showed that tumors with a p53 abnormality were more likely to be drug resistant than tumors with normal p53 (56% vs. 17%; p = 0.008). They were also more likely to have a shorter median progression-free survival (PFS) (2.1 vs. 8.2 months; p = 0.008) and overall survival (11.7 months vs. 21.5 months; p = 0.038) respectively. There were no significant associations between the presence of a p53 abnormality and patient characteristics such as age, LDH level, ECOG performance status, stage, extranodal site, or histology. This study clearly establishes the importance of a p53 abnormality as a possible prognostic factor in NHL. One explanation for
the lack of association between a p53 abnormality and other clinical indices is that unlike the previous study which looked at previously untreated cases of B-cell lymphoma, the patient population of this study comprised patients with relapsed refractory NHL. This study had therefore already selected for aggressive/refractory cases of NHL, and as such reflected the clinical indices of the International Prognostic Index (older age, presence of extranodal disease, elevated LDH, higher stage, and higher ECOG status).

In another study mutations in p53 were identified in 25 of 237 patients (10.5%) (321). Clinical data were available for 66 patients, 17 of whom had been shown to have p53 mutations. Twenty two of 49 patients without mutation died within 1 to 68 months (median – 13 months), whereas 14 of 17 patients with a mutation died within 1 to 88 months (median – 12 months) (P<0.05). As well, fifty patients had their tumor samples examined for p53 expression. Twenty six patients were positive for p53 expression and twenty four patients were negative for p53 expression. Of the 26 p53 positive patients, 21 died within 1 to 68 months (median – 13 months), and five were alive at a median follow-up of 33 months. Among the 24 p53 negative patients, one patient died at 63 months, and 23 were alive at a median follow-up duration of 46 months. There was a statistically significant difference between the two groups (p<0.001). The results of this study again show that p53 mutation and/or expression has a negative influence on survival.

In a fourth study of fifteen non-villous splenic marginal zone lymphoma patients, six cases showed p53 mutations (40%) (331). After a median follow-up of 56 months, four cases evolved into aggressive fatal NHL, and three of these demonstrated p53 mutations at diagnosis. Two cases
had refractory progressive disease, and both of these cases had p53 mutations at diagnosis. Of the nine unmutated cases eight had indolent or responsive disease and the remaining patient evolved into a Burkitt-like NHL. These results support the hypothesis that a p53 mutation increases the likelihood of transformation to a more aggressive phase.

In conclusion, all these studies have found the presence of p53 mutation to be a predictor of poor outcome (Table 10).

Several studies have examined p53 expression and its correlation with prognosis. In one study, samples from 44 NHL patients were analyzed for p53 expression in order to delineate if p53 expression was correlated with resistance to cytotoxic drugs (332). Patients were staged after clinical examination and all stages (I-IV) were present in the study population. All samples were obtained at diagnosis except for one patient who was studied in primary refractory disease. Patients were treated with different polychemotherapy programs.

Patients in whom the p53 protein was present had a significantly poorer prognosis with no objective response after chemotherapy (0/9 patients), as compared with the patients from the p53 negative subgroup (27 responders from 35 p53 negative patients, p < 0.001). The weakness of this study is that all the patients did not receive the same chemotherapy regimen and therefore comparison between the groups is difficult.

In a study by Korkolopoulau et al. (333) ninety two patients with NHL (low, intermediate,
and high-grade) were assessed for p53 protein expression using the Do-1 antibody. Follow-up information was available for eighty nine patients. Specimens were considered p53 positive when an unequivocal nuclear staining beyond background was seen in at least 0.1% of neoplastic cells. Other parameters that were assessed included the proliferative rate by way of the percentage of ki67 positive cells, the apoptotic index using TUNEL (terminal deoxynucleotidyl-transferase mediated in-situ end-labeling technique), as well as clinical indices (age, stage, bone marrow involvement, presence or absence of B symptoms, and location of the tumor (nodal vs. extranodal). Patients were followed until death, or for an average of 63 months. In multivariate analysis p53 protein expression independently predicted for poor overall survival (p = 0.0061) and a lower probability of complete remission (p = 0.0075).

In another retrospective study of fifty patients with aggressive NHL treated with CHOP chemotherapy, patients were stratified according to the International Index (334). The predictive value of p53 tumor suppressor protein was assessed. P53 expression was measured immunohistochemically using the Do-7 monoclonal antibody. Tumor samples were considered negative for p53 expression if <5% of the neoplastic cells exhibited nuclear staining. Forty percent of the lymphomas had ≥5% of the cells staining positively for p53 and this finding correlated significantly with response to treatment. The overall survival was significantly shorter (17 months) in p53 positive patients, as compared to the p53 negative group (>24 months) (p = 0.003). Furthermore, sixty six percent of patients in the p53 negative group remained in complete remission one year following completion of therapy in comparison with only 20% of patients in the p53 positive group (p = 0.001).
In another study the prognostic value of proliferating cell nuclear antigen (PCNA), and p53 protein expression, in relation to classic clinicopathological parameters was assessed in ninety-one untreated patients with NHL (low intermediate, and high-grade) (1). The cut-off for p53 positivity was 0.1% of the cells. Clinical parameters that were assessed included age, location (nodal/extranodal), clinical stage, bone marrow involvement, B-symptoms, and treatment (treatment with radiotherapy (yes/no), and the type of chemotherapy). The follow-up period lasted 4 to 193 months (median 48 months). In multivariate analysis p53 protein expression was nearly statistically significant in independently predicting post-relapse survival (p = 0.0973), but was not statistically significant in predicting overall survival, or disease-free survival.

In a study by Sakai et al., tissue from thirty-five patients with fresh and relapsed NHL was analyzed for expression of p53 (335). The cases included low, intermediate, and high-grade NHL according to the WF. All patients were treated with CHOP or a more intensive protocol composed of an alternating anthracyclin-containing regimen. Cells with greater than 3% nuclear staining were considered p53 positive. The ratio of partial remission, progressive disease or complete remission between the p53 positive and negative patients did not differ significantly among aggressive NHL (p = 0.721). Low-grade NHL was not assessed because all of these patients were p53 negative. As well, p53 expression did not correlate with high risk parameters of the International Index.

In conclusion, although most reports have found p53 protein expression to be statistically significant in independently predicting overall survival and disease-free survival (334, 335, 336),
some reports (1, 335) have failed to find this correlation (Table 10). One explanation for this discrepancy is inter-lab variation in assessing p53 positivity (Table 9). Furthermore, as described in the introduction, immunohistochemistry is not highly specific in NHL, as there have been several cases in which positive cells by immunohistochemistry did not show p53 mutations by SSCP or direct sequencing (308). The preferred method for p53 mutation detection is SSCP combined with direct sequencing. Therefore, the studies already described which assessed p53 mutation using SSCP and direct sequencing are more noteworthy. These studies all found a correlation between p53 mutation and poor outcome.
<table>
<thead>
<tr>
<th>Author (Reference)</th>
<th>Retrospective vs. Prospective</th>
<th>Previously Treated vs. Untreated</th>
<th># of Patients</th>
<th># with Normal p53 Gene</th>
<th># with Mutated p53</th>
<th># with No p53 Expression</th>
<th># with p53 over-expression</th>
<th>Survival/Complete Remission/ Drug Resistance</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ichikawa A. (323)</td>
<td>Prospective</td>
<td>Untreated Intermediate or high-grade B-Cell NHL</td>
<td>102</td>
<td>80</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>6/22 patients with mutated p53 had CR Kaplan-Meier estimate of survival at 5 years 16% Kaplan-Meier estimate of survival at 5 years 64%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Wilson W. (322)</td>
<td>Prospective</td>
<td>Previously Treated (relapsed/refractory NHL)</td>
<td>75</td>
<td>42 (56 pts assessed for p53 mutation)</td>
<td>14</td>
<td>62 (75 patients assessed for p53 over-expression)</td>
<td>13</td>
<td>56% of tumors with p53 abnormality were drug resistant 11.7 month overall survival for patients with a p53 abnormality 2.1 month median progression-free survival for patients with a p53 abnormality</td>
<td>0.008</td>
</tr>
<tr>
<td>Koduru P. (321)</td>
<td>Retrospective</td>
<td>Previously treated and untreated</td>
<td>237</td>
<td>212</td>
<td>25</td>
<td>-</td>
<td>-</td>
<td>Clinical data available for 17 patients with a mutation, 14/17 patients with a mutation died within 1-88 months (median - 12 months) Clinical data available for 49 patients with normal p53, 22/49 patients died within 1-68 months (median - 13 months)</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21/26 p53 +ve patients died within 1-68 months (median 13 months), and 5 were alive at a median follow-up of 33 months 1/24 p53 -ve patients died at 63 months, and 23 were alive at a median follow-up of 46 months</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

-77-
<table>
<thead>
<tr>
<th>Author</th>
<th>Retrospective vs. Prospective</th>
<th>Previously Treated vs. Untreated</th>
<th># of Patients</th>
<th># With No p53 Expression</th>
<th># With p53 expression</th>
<th>Survival/CR/Drug Resistance</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sun R.X. (332)</td>
<td>Retrospective</td>
<td>Untreated and Treated</td>
<td>44</td>
<td>35</td>
<td>9</td>
<td>0/9 patients had no objective response after chemotherapy</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Korkolopolau P. (333)</td>
<td>Retrospective</td>
<td>F/U-until death or for an average of 63 months</td>
<td>92 - F/U information available for 89</td>
<td>65</td>
<td>27</td>
<td>Multivariate analysis showed that p53 expression is correlated with shorter overall survival</td>
<td>0.0061</td>
</tr>
<tr>
<td>Navaratnam S. (334)</td>
<td>Retrospective</td>
<td>Untreated</td>
<td>50</td>
<td>30</td>
<td>20</td>
<td>17 month overall survival</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20% remained in complete remission 1 year following completion of therapy</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;24 month overall survival</td>
<td></td>
</tr>
<tr>
<td>Korkolopolau P. (1)</td>
<td>Retrospective</td>
<td>Untreated</td>
<td>91</td>
<td>48</td>
<td>43</td>
<td>In multivariate analysis, p53 expression independently predicted post-relapse survival, but not overall survival or disease-free survival</td>
<td></td>
</tr>
<tr>
<td>Sakai A. (335)</td>
<td>Prospective</td>
<td>Untreated and Treated</td>
<td>35</td>
<td>23</td>
<td>12</td>
<td>p53 expression did not predict early response to chemotherapy</td>
<td>0.721</td>
</tr>
</tbody>
</table>
FUTURE DIRECTIONS

The results of this thesis have found a statistically significant correlation between having a p53 mutation and elevated LDH levels, and a decreased overall survival. Having a p53 mutation was also shown to be nearly statistically significant in correlation with aggressive (intermediate and high grade) disease. The work described in this thesis could be elaborated on in several ways in the future. To begin with, the mutant samples could be sequenced for definitive analysis. In addition, to have a more comprehensive SSCP analysis, exon 4 of the p53 gene can also be analyzed. As well, multivariate analysis could be carried out to examine if having a p53 mutation is an independent prognostic factor when compared with other predictors of poor survival in NHL.

Repeated intratumoral injection of the adenoviral p53 vector (Ad5CMV-p53) in patients with non-small cell lung cancer and head and neck cancer has been shown to be well tolerated (337). This technique could be used in patients with NHL who have been shown to have p53 mutations.

Finally, the method of p53 mutation detection described here could be used to assess other genes involved in the cell cycle, and apoptotic pathways to determine if a mutation in these genes will correlate with poor survival.
REFERENCES


11. NHL Pathology Classification Project: National Cancer Institute Sponsored Study of Classification of NHL; Summary and description of a Working Formulation for Clinical Usage. Cancer 49: 211, 1982


-80-


47. Bastard C, Tilly H, lenormand B, Bigorgne C, Boulet D, Kunlin A, Monconduit M, Piguet
H: Translocations involving band 3q27 and Ig gene regions in NHL. Blood 79(10): 2527-2531, 1992


59. Prochownk EV, Kukowska J, Rodgers C: C-myc antisense transcripts accelerate differentiation and G1 progression in murine erythroleukemia cells. Molecular and Cellular Biology 8(9): 3683-3695, 1988


69. Hockenberry D, Nunez G, Milliman C: Bcl-2 is an inner mitochondrial membrane protein


Investigation 82(1): 370-372, 1993


156. Hupp TR, Meek DW, Midgley CA, Lane DP: Regulation of the specific DNA binding function of p53. Cell 71(5): 875-886, 1992


176. Ko LJ, Shieh SY, Chen X, Jayaraman L, Tamai K, Taya Y, Prives C, Pan ZQ: P53 is
phosphorylated by CDK7-cyclin H in a p36MAT1-dependent manner. Molecular and Cellular Biology 17(12): 7220-7229, 1997

177. Bischoff JR, Friedman PN, Marshak Dr, Prives C, Beach D: Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. Proceedings of the National Academy of Sciences of USA, 87(12): 4766-4770, 1990


188. Sakaguchi K, Herrera JE, Saito S, Miki T, Bustín M, Vassilev A, Anderson CW, Appella E: DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes and
Development 12(18): 2831-2841, 1998


Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB: Wild-type p53 is a cell cycle check-point determinant following irradiation. Proceedings of the National Academy of Sciences of the USA, 89(16): 7491-7495, 1992


233. DeGregori, Kowalik T, Nevins JR: Cellular targets for activation by the E2F1 transcription factor include DNA synthesis and G1/S synthesis and G1/S regulatory genes. Molecular and Cellular Biology 15(8): 4215-4224


expression upon genotoxic stress correlates with reduced binding of free E2F to the promoter. Cell Growth and Differentiation 8(16): 699-710, 1997


274. Bi S, Lanza F, Goldman JM: The abnormal p53 proteins expressed in CML cell lines are
non-functional. Leukemia 7(11): 1840-1845, 1993


278. Farrugia M, Duan LJ, Reis MD, Ngan BY, Berenstein NL: Alterations of the p53 tumour suppressor gene in diffiuse large cell lymphomas with translocations of the c myc and Bcl-2 proto-oncogenes; Blood 83(1): 191-198, 1994


based technique. Leukemia 7(4): 593-600, 1993


308. Detournigines L, Copin MC, Morel P, Venrumbeke M, Preudhomme C, Wattel E: P53 overexpression in diffuse large cell lymphoma (DLCL) and its prognostic value by


320. Friedman PN, Chen X, Bargonetti J, and Prives C: The p53 protein is an unusually shaped tetramer that binds directly to DNA. Proceedings of the National Academy of Sciences USA 90(8): 3319-3323, 1993


343. Kim YS., Kim JB., Kang YK., Nam ES., Park SH., Kim I., Viral genotypes and p53 expression in Epstein-Barr virus-associated primary malignant lymphomas of the intestines,
Human Pathology, 30 (10), 1146-52, 1999
