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# Molecular Genetic Analysis in B-cell Lymphomas

## *A Focus on the p53 Pathway and p16$^{INK4a}$*

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**Abstract**
Zainuddin, N. 2010. Molecular Genetic Analysis in B-cell Lymphomas. A Focus on the p53 Pathway and $p16^{INK4a}$. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 525. 78 pp. Uppsala. ISBN 978-91-554-7729-5.

The presence of *TP53* mutations has been associated with inferior outcome in diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL). In DLBCL, the impact of the *TP53* codon 72 polymorphism and *MDM2* SNP309 has not been clearly elucidated, whereas *MDM2* SNP309 was suggested as a poor-prognostic marker in CLL. In addition, $p16^{INK4a}$ promoter hypermethylation has been implicated as a negative prognostic factor in DLBCL. The aim of this thesis was to further evaluate these molecular markers in well-characterised materials of DLBCL and CLL.

In paper I, we investigated the prognostic role of *TP53* mutation, codon 72 polymorphism and *MDM2* SNP309 in DLBCL (n=102). The presence of *TP53* mutations (12.7%) correlated with a poor lymphoma-specific and progression-free survival, and a particularly pronounced effect was observed in the germinal center subtype. Neither the *MDM2* SNP309 nor the *TP53* codon 72 polymorphism had an impact on age of onset or survival. In paper II, we applied pyrosequencing to measure the level of $p16^{INK4a}$ methylation in DLBCL (n=113). Thirty-seven percent of cases displayed $p16^{INK4a}$ methylation; however, no clear association could be observed between degree of methylation and clinical characteristics or lymphoma-specific survival.

In papers III–IV, we investigated the prognostic role of *MDM2* SNP309 (n=418) and *TP53* mutation (n=268) in CLL. No correlation was observed between any particular *MDM2* SNP309 genotype and time to treatment and overall survival. Furthermore, no association was found between the different *MDM2* SNP309 genotypes and established CLL prognostic markers. *TP53* mutations were detected in 3.7% of CLL patients; where the majority showed a concomitant 17p-deletion and only three carried *TP53* mutations without 17p-deletion. We confirmed a significantly shorter overall survival and time to treatment in patients with both *TP53* mutation and 17p-deletion.

Altogether, our studies could confirm the negative prognostic impact of *TP53* mutations in DLBCL, whereas *MDM2* SNP309 and *TP53* codon 72 polymorphisms appear to lack clinical relevance. We also question the role of $p16^{INKa}$ methylation as a poor-prognostic factor in DLBCL. Finally, the presence of *TP53* mutation in CLL appears to be rare at disease onset and instead arise during disease progression.

*Keywords:* Diffuse large B-cell lymphoma, chronic lymphocytic leukemia, TP53 mutation, MDM2 SNP309, codon 72 polymorphism, p16INK4a methylation

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To my mother,
Norsham Abdul Majid

*~All that I am and I ever hope to be,*
*I owe to my mother~*
*Abraham Lincoln*

# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Zainuddin N**, Berglund M, Wanders A, Ren ZP, Amini RM, Lindell M, Kanduri M, Roos G, Rosenquist R, Enblad G. *TP53* mutations predict for poor survival in *de novo* diffuse large B-cell lymphoma of germinal center subtype. *Leukemia Research* 2009; 33(1):60-6.

II **Zainuddin N***, Kanduri M*, Berglund M, Lindell M, Amini RM, Roos G, Sundström C, Enblad G, Rosenquist R. Quantitative evaluation of $p16^{INK4a}$ promoter methylation using pyrosequencing in *de novo* diffuse large B-cell lymphoma. *Manuscript*.
*Contributed equally as first authors

III Kaderi MA, Mansouri M, **Zainuddin N**, Cahill N, Gunnarsson R, Jansson M, Kimby E, Åleskog A, Lundin J, Glimelius B, Melbye M, Juliusson G, Jurlander J, Rosenquist R. Lack of association between the *MDM2* SNP309 and clinical outcome in chronic lymphocytic leukemia. *Leukemia Research* 2010; 34(3):335–9.

IV **Zainuddin N***, Murray F*, Kanduri M*, Gunnarsson R, Smedby KE, Enblad G, Julander J, Juliusson G, Rosenquist R. *TP53* mutations are infrequent in newly-diagnosed chronic lymphocytic leukemia. *Manuscript*.
*Contributed equally as first authors

# Contents

# Abbreviations

| | |
|---|---|
| ABC | Activated B-cell like |
| ATM | Ataxia telangiectasia mutated |
| BCR | B-cell receptor |
| CHOP | Cyclophosphamide, doxorubicin, vincristine, prednisone |
| CLL | Chronic lymphoctic leukemia |
| DLBCL | Diffuse large B-cell lymphoma |
| DNA | Deoxyribonucleic acid |
| EBV | Epstein-Barr virus |
| FISH | Fluorescence *in situ* hybridization |
| FL | Follicular lymphoma |
| GC | Germinal center |
| GEP | Gene expression profiling |
| IG | Immunoglobulin |
| IGH | Immunoglobulin heavy chain |
| IGHV | Immunoglobulin heavy chain variable |
| IPI | International Prognostic Index |
| LSS | Lymphoma-specific survival |
| MALT | Mucosa-associated lymphoid tissue |
| MCL | Mantle cell lymphoma |
| MDM2 | Murine double minute 2 |
| MSP | Methylation specific PCR |
| MZL | Marginal zone lymphoma |
| NF-κB | Nuclear factor kappa B |
| PcG | Polycomb group |
| PCR | Polymerase chain reaction |
| PFS | Progression-free survival |
| PMBL | Primary mediastinal B-cell lymphoma |
| $PP_i$ | Pyrophosphate |
| R | Rituximab |
| RFLP | Restriction fragment length polymorphism |
| SHM | Somatic hypermutation |
| SNP | Single nucleotide polymorphism |
| V | Variable |
| WHO | World Health Organization |

# INTRODUCTION

## B-cell Lymphomas and Leukemias

Approximately 20 new cases of lymphoma are diagnosed per 100,000 individuals in the Western countries every year[1]. According to the Swedish Cancer Registry, more than 2000 new lymphoma and leukemia cases are diagnosed annually in Sweden[2]. The incidence rate of lymphoma has increased with about 4% every year up until the last decade[3], whereafter the rate has subsided in the Nordic countries[4].

Development of lymphoma is initiated by a genetic alteration in a cell that predisposes it to undergo further genetic alterations. The additional acquired abnormalities later promote the development of a clone with growth or survival advantage over other cells. This will eventually develop into a clinical lymphoma. However, what triggers these genetic alterations is largely unknown. Although several aspects of the multistep processes leading to lymphomagenesis have been discovered, most contributing mechanisms underlying transformation are still unknown. Nevertheless, as research accelerates, several risk factors for lymphoma have been established. A history of hematopoietic malignancy in first-degree relatives has been correlated to an increased risk of B-cell lymphomas, with stronger aggregation for siblings[5,6]. Moreover, evidence suggests that viral infections, such as Epstein-Barr virus (EBV) and hepatitis C virus, as well as bacterial infections, such as *Chlamydophila psittaci* and *Borrelia burgdorferi* can contribute to lymphomagenesis[7,8]. The risks of developing lymphomas also increases in patients with compromised immune system due to HIV infection, autoimmune disease or treatment with immunosuppressive drugs following an organ transplant[9]. In addition, exposure to certain chemicals such as benzene, herbicides and pesticides, and radiations has been linked to risks of developing lymphoma[10].

Lymphomas are very heterogeneous, both from biological and clinical perspectives. The World Health Organization (WHO) has thus classified lymphomas based on a combination of morphology, immunophenotype, genetic and clinical features. In general, lymphomas are divided into mature B-cell lymphomas, T- and NK-cell lymphomas, and Hodgkin lymphomas[11]. About 85% of all lymphomas are B-cell lymphomas, which include diffuse large B-

cell lymphoma (DLBCL), follicular lymphoma (FL), chronic lymphocytic leukemia (CLL), extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT), mantle cell lymphoma (MCL) and Burkitt's lymphoma. However, this thesis will be focusing on DLBCL and CLL.

DLBCL is an aggressive but potentially curable disease, with 70% to 80% of complete remissions achieved after chemotherapy or radiotherapy. On the other hand, CLL is generally considered an indolent disease where many patients are asymptomatic at diagnosis. CLL patients may survive for many years without treatment, but because of the low proliferative disease, the majority of patients who respond to current treatment will eventually relapse. However, a significant proportion of CLL patients will also follow an aggressive course of disease, despite initiation of treatment.

## Normal B-cell Development and IG Gene Rearrangements

B-cell lymphomas arise from normal lymphocytes at distinct differentiation steps of B-cell development. The first stage of B-cell development is antigen-independent and takes place in the bone marrow, where random recombination of variable (V), diversity (D) and joining (J) gene segments at the immunoglobulin heavy (IGH) and light chain locus occurs[12]. Once the B-cell carries functional heavy and light chain rearrangements, it expresses a complete Ig molecule, along with accessory molecules on its surface[13]. This structure is known as the B-cell receptor (BCR). Immature B-cells that express a functional BCR then leave the bone marrow and migrate to the peripheral blood, where they become transitional B-cells and complete their maturation into naïve B-cells[14]. Naïve B-cells are capable of interacting with antigens, which are required for the cells to become activated[15]. With the help of T-cells, antigen-activated B-cells migrate into primary B-cell follicles in the lymphoid organs and undergo clonal expansion in structures called germinal centers (GCs) (Figure 1). The next stage of B-cell maturation process takes place in the GC, where the IG genes are modified by somatic hypermutation (SHM) and class-switch recombination[16]. SHM takes place in the dark zone of the GC, where mutation of the IG V regions occurs[17]. Most mutations are disadvantageous for the cell, such as those that lead to reduced affinity of the BCR for antigen. These cells will eventually die by apoptosis. Others will acquire mutations in the BCR which will improve the affinity for antigen, and these cells will be selected for further differentiation. This selection process takes place mainly in the light zone, where the B-cells are in close contact with $CD4^+$ T-cells and follicular

dendritic cells. A proportion of the selected B-cells then undergo class-switch recombination, which changes their isotypes, thus allowing the production of antibodies with distinct effector functions[18]. Finally, the GC B cells differentiate into long-lived memory B-cells or plasma cells which secrete Ig, and leave the GC microenvironment[19].

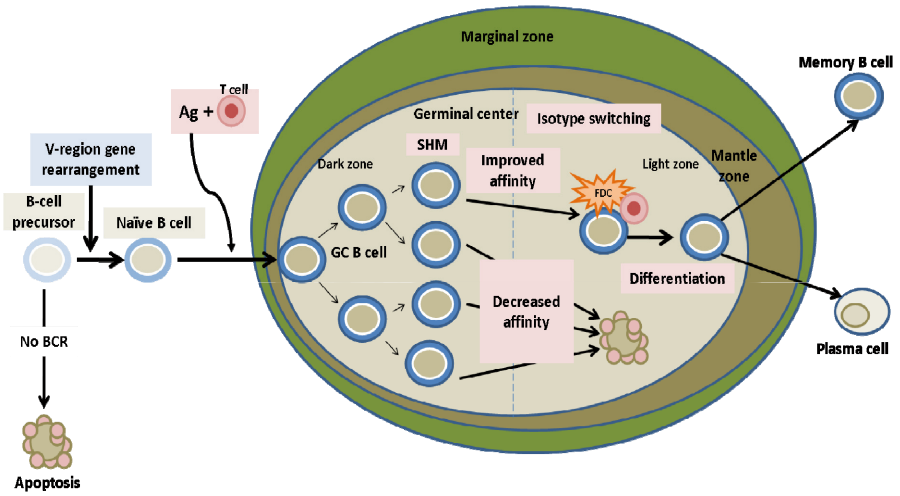


*Figure 1.* The GC reaction. Modified from Kuppers[20].

## Cellular Origin of B-cell Lymphomas

According to the current model of lymphomagenesis, most of the recognized entities of B-cell lymphomas/leukemias can be traced to particular stage(s) of B-cell differentiation, although the extent to which these malignancies maintain the molecular and physiological properties of certain normal B-cell subsets is not fully elucidated. B-cells that have undergone VDJ recombination carry specific heavy and light chain IG gene rearrangement with or without presence of SHM. Whenever a B-cell undergoes malignant transformation and clonal proliferation, each daughter cell will carry identical IG gene rearrangements. Thus, IG gene rearrangements provide useful information on the origin and clonal history of the B-cell tumors[20,21]. Moreover, the presence of somatic mutations in the V region of the IG genes will indicate whether the cell of origin has undergone SHM and affinity maturation under the influence of antigen (Figure 1).

DLBCL are examples of GC or post-GC derived B cell malignancies which contain somatically hypermutated IG genes[22]. The presence of distinct DLBCL subtypes with different gene expression patterns was initially shown by gene expression profiling (GEP) analysis[23,24] (described in other section of this thesis). The GC DLBCL subtype expresses genes that are characteristic of normal GC B-cells, suggesting its GC origin[23,24]. On the other hand, activated B-cell like (ABC) tumors express genes characteristic of *in vitro* activated peripheral blood B-cells, as well as genes which are normally expressed by plasma cell, suggesting its post-GC origin[23,24]. The presence of ongoing SHMs occurs primarily in the GC DLBCL subtype[22], further supporting its GC origin, whereas ongoing SHM cannot be found in the non-GC subtype[22].

The cellular origin of CLL is still obscure, and different candidates have been suggested over the years. Currently, there is no normal B-cell population that shares the unique immunophenotype of CLL cells. Thus, a direct assignment of a normal B-cell population as a normal counterpart for CLL is difficult. CLL was originally thought to arise from pre-GC B cells, possibly arising in the follicular mantle zone, with no evidence of mutations within the IG genes[25]. The expression of the CD5 antigen suggested that CLL originates from resting $CD5^+$ B-cells[26], which are usually characterized by unmutated IGHV genes[27,28]. However, studies have shown that 50–70% of CLL undergone SHM[29-31]. This finding has led to the hypothesis that there may be two separate entities of CLL; one with unmutated IGHV genes, originating from pre-GC B-cells, and one with mutated IGHV, which originates from post-GC B-cells[32,33]. GEP has later shown that both IGHV mutated and unmutated CLL have a homogenous expression profile similar to memory cells[34,35]. Moreover, immunophenotyping indicated that all CLL cases, including those with unmutated IGHV gene, resemble antigen-experienced B-cells, and that the CLL cells exhibit an activated state[36]. Recently, it has also been proposed that the unmutated and mutated CLL cells might actually arise from the marginal zone B-cells that are triggered independent of T-cells[37].

## Transforming Events in B-cell Lymphoma Pathogenesis

The transforming events in the pathogenesis of B-cell lymphomas mainly occur at two stages: during the random recombination of VDJ gene segments in the bone marrow, and during the transit of B-cells through the GC. During these stages, reciprocal translocations involving one of the IG loci and a proto-oncogene can occur[38,39]. As a result of the translocation, the oncogene is brought under control of active IG transcriptional elements, causing a

constitutive expression of the oncogene. These illegitimate recombinations follow double-strand DNA breaks, which are generated during VDJ recombination, SHM and class switch recombination. For example, translocations of *BCL2*, *BCL6* and *MYC* oncogene to the IGH locus lead to the up-regulation of these oncogenes[40-42] (see below). Chromosomal translocations can involve not only IG gene loci, but also non-IG chromosomal loci as a partner[43-45]. However, it should be noted that the sole presence of these translocations is not sufficient to induce lymphomagenesis, and additional genetic hits, or "secondary genetic events", are necessary for lymphoma development and progression[39]. Other transforming events implicated in the pathogenesis of B-cell lymphomas include, for instance, mutations of the *TP53* gene and aberrations of the p53 pathway[46,47], inactivation of the ataxia telangiectasia mutated (*ATM*) gene by deletions and mutations[48,49], inactivation of the $p16^{INK4a}$ gene by deletion and hypermethylation[50,51], and genomic amplifications of *REL*, *C-MYC* and *BCL2*[52].

Recently, a study by Martin-Subero *et al.*[53] has provided a new theory on the development of mature aggressive B-cell lymphoma, including DLBCL. In contrast to the commonly-accepted genetic model of lymphomagenesis, they have suggested three different alternative pathways that might initiate lymphomagenesis: 1) Aberrant DNA methylation of polycomb group (PcG) target genes in stem or progenitor cells. PcG proteins are transcription regulatory proteins that are thought to repress gene transcription. 2) Chromosomal aberrations in stem or precursor cells. The stem cell carrying a chromosomal translocation would then acquire aberrant DNA methylation of PcG target genes. 3) Chromosomal aberrations in a differentiating cell. Due to deregulation of genes caused by translocations, this differentiating cell would then acquire stem-cell like features, and subsequently acquire aberrant DNA methylation of PcG target genes. Finally, additional genetic and epigenetic hits would result in the development of a mature, aggressive B-cell lymphoma.

# Diffuse Large B-cell Lymphoma

## Epidemiology and Etiology

DLBCL constitutes the largest category of aggressive lymphomas, accounting for 30% to 40% of all new cases of B-cell lymphomas in the Western countries each year[54]. The median age at diagnosis is 65 years, although this lymphoma can occur in children and young adults. Men are slightly more commonly affected than women.

DLBCL is a clinically and biologically heterogeneous disease, where the etiology remains largely unknown. It usually arises *de novo* (primary), which is without a previous history of an indolent lymphoma, or as transformed (secondary) DLBCL originating from a less aggressive lymphoma, for example FL, CLL, or marginal zone lymphoma (MZL). A significant risk factor in DLBCL is immunodeficiency, which is commonly associated with EBV infection[7], and autoimmune disease[55].

## Tumor Pathology

### Morphology

In DLBCL, the term diffuse refers to the fact that the tumor cells are spread around in one particular part of the node or in clusters within a part of the node. Histologically, the lymph nodes are usually entirely effaced, and the perinodal tissue is frequently infiltrated. Morphologically, this entity is characterized by a diffuse proliferation of large lymphoid cells, with vesicular nuclei and prominent nucleoli, which include centroblasts, immunoblasts and anaplastic large B-cells[11]. The centroblastic variant is the most common morphologic variant of DLBCL. Centroblasts are medium to large lymphoid cells with oval to round vesicular nuclei containing fine chromatin. The cytoplasm is usually scanty, and many mitotic and apoptotic cells are usually present (Figure 2).

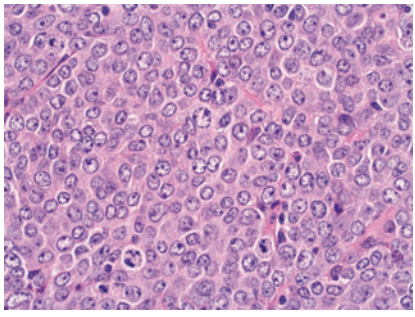

*Figure 2* The centroblastic variant of DLBCL.

### Immunophenotype

The tumor cells express CD19, CD20, CD22 and CD79a, which are the typical B-cell markers, and surface and/or cytoplasmic Ig (IgM>IgG>IgA) is present in 50% to 75% of cases[11]. The incidence of CD10, Bcl-6 and MUM1/IRF4 expression in DLBCL varies in different studies (described in other section of this thesis). The proliferation fraction, measured as Ki67 expression, is usually high in this disease and an index of 80% or greater has been shown to correlate with poor outcome[11].

### Sites of Involvement

Nodal and extranodal presentation is common in DLBCL patients, with almost one-third of these patients have disease confined to extranodal sites at the time of diagnosis[56]. The most common extranodal presentation is in the gastrointestinal tract, but involvement of other sites including bone, spleen, salivary glands and liver has been reported[11].

## Classification and Prognostic Factors

### The International Prognostic Index

DLBCL patients may have markedly divergent clinical courses and treatment response. Attempts to identify subgroups of DLBCL based on morphology have largely failed due to diagnostic discrepancies of inter- and intra-observer reproducibility[57,58]. Since its publication in 1993, the clinical prognostic factors described in the International Prognostic Index (IPI) have been used in risk stratification for patients with DLBCL[59]. This classification is based on five factors; age, serum lactate dehydrogenase level, performance status, clinical stage and number of extranodal sites. According to this system, DLBCL patients could be categorized into low risk, intermediate low risk, intermediate high risk and high risk groups, all of which have different rates of complete response, relapse-free survival and overall survival. The subsequent age-adjusted (aa)-IPI, which is based on gender and stage of disease, has also been applied extensively. Although it could be used to prognostically identify young patients at risk (<60 years of age) treated with CHOP (see below), the aa-IPI no longer remains prognostic in patients treated with R-CHOP (see below)[60]. The relevance of IPI in R-CHOP treated patients was later tested, which led to the redistribution of the four IPI risk factors into three risk groups based on outcome. This revised (R)-IPI categorized the patients into “very good”, “good” and “poor” risk groups[61]. R-IPI has proved valuable for stratification of patients in clinical trials. However, neither the IPI nor R-IPI is predictive in patients with less than 50% chance of survival.

Though well-established, it is obvious that IPI, aa-IPI and R-IPI fail to predict patients’ outcome in a proportion of cases. The emergence of GEP has significantly altered the approach to classify and predict prognosis in DLBCL, as it unravelled distinct subgroups of DLBCL independent from the IPI classification. The application of GEP for categorizing DLBCL is further described in the next section.

### Distinct DLBCL subtypes

GEP has successfully been applied to categorize many lymphoma entities, and has revealed the molecular heterogeneity that exists within diagnostic

categories. Indeed, using GEP, DLBCL could be distinguished into two major molecular subtypes according to profiles resembling normal B-cells; the GC and ABC DLBCLs[23,24]. Alizadeh *et al.*[23] have shown that GC DLBCL patients had significantly better overall survival than patients with ABC DLBCL. These findings were confirmed by Rosenwald *et al*[24], who have also identified type 3 DLBCL, a heterogeneous subtype that does not express high levels of either the GC or ABC set of genes. Since patients with type 3 DLBCL showed similar clinical outcome as patients with the ABC subtype, Hans *et al.*[62] grouped both subtypes as non-GC DLBCL. Moreover, Hans *et al.*[62] and Haarer *et al*.[63] have shown that it is possible to use immunohistochemical staining to subdivide DLBCL into GC and non-GC subtypes. Based on the cellular origin of B-cells and expression of CD10, Bcl-6 and MUM1/IRF4 proteins[62,63], they have demonstrated that immunohistologic model is able to subclassify DLBCL and predict patient survival similar to those predicted by GEP. The findings by Hans *et al.*[62] were later confirmed in an independent DLBCL material by Berglund *et al.*[64]. Nevertheless, the value of the immunohistochemical model has been questioned since it does not seem to work on patients treated with rituximab containing chemotherapy (see below) [65], whereas the GEP model still gives prognostic information in that group[24]. Recently, Choi *et al.*[66] have demonstrated a new immunohistochemistry algorithm based on GCET1, CD10, Bcl-6, MUM1 and FOXP1 protein expression, which had a 93% concordance with the GEP classification of DLBCL. This new algorithm has shown to be more accurate in classifying patients' prognosis compared to the algorithm proposed by Hans *et al.*[62].

Another important but uncommon subtype of DLBCL has been identified by Rosenwald *et al.*[67] and Savage *et al.*[68]: the primary mediastinal B-cell lymphoma (PMBL). PMBL patients are younger, and show better overall survival than ABC DLBCL patients, but only slightly better than patients with the GC subtype[67]. Choi *et al.*[66] have analyzed seven PMBL cases in their study, and reclassified PMBL as "GC" according to their new algorithm, which differs from the classification by Hans *et al.*[62] (two "GC" and five "non-GC"). The reclassification of PMBL cases as "GC" is an improvement over the algorithm by Hans *et al.*[62], and is considered acceptable for prognostication as both PMBL and GC DLBCL patients showed good prognosis[67].

Despite the classification of the major DLBCL subtypes by GEP and the application of the immunohistologic model, this disease still remained heterogeneous regarding treatment response and survival. Given that the biologic processes in lymphoma development involve multiple genes, signaling pathways and regulatory mechanisms, it is not surprising that no single

marker is sufficient to accurately confine the heterogeneity of DLBCL. Recognizing this, other predictive markers in DLBCL have been identified: the gene expression signatures including the proliferation, lymph node, and MHC class I and II signatures[24], the BCR/proliferation, host response, and oxidative phosphorylation signatures[69], and the stromal I and II signatures[70].

**Genomic aberrations**

Genomic alterations in the form of translocations or point mutations have been implicated as prognostic factors in DLBCL. The t(14;18) translocation, involving the *BCL2*-IGH genes, is detected in 12% to 30% of DLBCL[41,71,72]. Increased expression of Bcl2, which is caused by *BCL2* translocation, has been associated with an adverse prognosis in DLBCL[41,73]. *BCL6*-IGH translocations were observed in up to 41% of all DLBCL cases[41,74]. *BCL6* translocations can also involve non-IG chromosomal loci as partners, which occur at an equal frequency as the *BCL6*-IGH translocation[75]. To date, approximately 20 non-IG partner genes have been identified[43]. No consensus on the effect of *BCL6*-IGH translocations have been associated with prognosis of DLBCL, with studies showing either favorable[41] or unfavorable outcome[76] or no effect[77]. On the other hand, *BCL6*-non-IG translocation has been suggested as indicator of poor prognosis in DLBCL[43]. Translocation of *C-MYC* and IG genes is observed in up to 15% of DLBCL[40,78] and is often associated with an unfavorable prognosis[41,79].

*BCL2* translocation was frequently found in the GC type[73], although no association of the translocation with prognosis has been demonstrated[80]. A single study has shown that *BCL6* translocations occur at a higher frequency in the non-GC subtype (24%) compared to the GC subtype (10%)[75]. Despite this, high Bcl6 mRNA and protein expression is frequently detected in the GC subtype, which correlates with favorable outcome. *C-MYC* translocations have been shown in a single study to occur more frequently in GC DLBCL and predicts for poor outcome in this subtype[81]. Amplification of the *REL* locus on chromosome 2p have been associated, although not exclusively, with the GC subtype[82]. Gains of 12q12 were also frequently observed in GC DLBCL[83].

One of the most important features of the ABC subtype is the constitutive activation of nuclear factor kappa B (NF-κB). High expression of NF-κB target genes, including those that encode *BCL2*, *IRF4* and *cyclin D2* is frequently observed in ABC DLBCL. Inhibition of NF-κB has been shown to induce apoptosis and cell cycle arrest in ABC DLBCL cell lines[84]. Recently, the product of the *PRDM1* gene, or *BLIMP-1*, was proposed as a further important ABC marker, where it was reported to be frequently inactivated in ABC DLBCL[85]. Trisomy 3, 3q27 and 18q21-22 gains and 6q21-22 loss were

also commonly observed in this subtype[83]. t(14;18) is rarely detected in the ABC DLBCL[86]; however, frequent gain and amplification of *BCL2* and Bcl2 overexpression has been associated with unfavorable outcome in this subtype[87].

Mutations of the *TP53* tumor suppressor gene and hypermethylation-associated inactivation of $p16^{INK4a}$ have been demonstrated as adverse factors affecting survival in DLBCL. The significance of *TP53* mutation and aberration involving the genes in the p53 pathway, as well as the involvement of $p16^{INK4a}$ in DLBCL pathogenesis, will be further discussed in other sections of this thesis.

### Therapy and Outcome

At present, DLBCL treatments are based on chemotherapy and immunochemotherapy. The most widely used chemotherapy is the cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) regimen. With the CHOP-like chemotherapy, the survival rate of GC DLBCL patients was significantly higher than the survival rate of ABC DLBCL patients[23,24,88]. In recent years, rituximab (R), a chimeric anti-CD20 monoclonal antibody, has been incorporated into the treatment of DLBCL patients. The addition of rituximab to CHOP (R-CHOP) has significantly improved the survival of these patients compared to CHOP alone, particularly for those with poor prognostic factors such as non-GC cell origin[62], low Bcl-6[89], or high Bcl-2 expression[90]. Importantly, rituximab has been shown to improve complete response and overall survival rates in both the GC and non-GC subgroups[65,70,91,92]. The effectiveness of rituximab in the treatment of patients with the ABC (or non-GC) subgroup is probably by inhibiting the constitutive NF-κB signaling pathway[93]. Moreover, prolonged event-free and overall survival rates have been observed in elderly DLBCL patients treated with R-CHOP[94]. In contrast, those with advanced aa-IPI[61], *TP53* mutations[95], or lack of LMO2[96] still responded poorly to R-CHOP.

## Chronic Lymphocytic Leukemia

### Epidemiology and Etiology

CLL accounts for approximately 30% to 40% of all leukemias and is the most frequent type of leukemia among adults in Western countries[97]. Approximately 14,000 individuals were estimated to have prevalent CLL within the European Union member states in 2006[98]. In Sweden, 500 new cases are diagnosed each year[2]. Median age at diagnosis is 65 to 70 years, and its incidence in men is twice than reported for women.

The occurrence of CLL is not due to any known environmental factors, such as ionizing radiations or chemical compounds, and it is not frequently detected in patients with immunodeficiencies[99]. In contrast, a genetic and familial predisposition appears to be more relevant in the pathogenesis of CLL[100,101].

## Tumor Pathology

### Morphology

CLL is a leukemic, lymphocytic B-cell lymphoma which is distinguished from small lymphocytic lymphoma (SLL) by its leukemic appearance[102]. CLL results from an accumulation of mature-looking immunoincompetent lymphocytes in the blood, bone marrow, lymph nodes and spleen, where more than 99% of circulating CLL lymphocytes is in the $G_0$/early $G_1$ phase of the cell cycle[103]. CLL lymphocytes are generally smaller than normal and appear to be more fragile, resulting in the characteristic "smudge" cells (Figure 3).

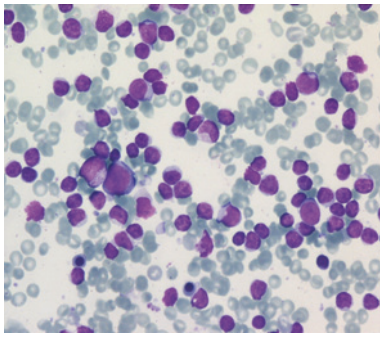

*Figure 3* Bone marrow smears showing CLL cells.

### Immunophenotype

CLL cells have a unique immunophenotype which are not found in any normal B-cell population. These cells co-express high levels of CD5 together with the B-cell surface antigens CD19, CD20 and CD23[104]. Moreover, the levels of surface IgM/IgD, CD20 and CD79b are characteristically low compared to those found in normal mature B cells[104]. CLL cells are also typically negative for the expression of FMC7, which in contrast is commonly expressed on prolymphocytic leukemia cells[105].

## Classification and Prognostic Factors

### Clinical staging systems

CLL is a disease with a highly variable course, mainly divided into two subclasses. The first consists of patients in whom the disease has an indolent course with no need for therapy, whereas the second class consists of patients suffering from a more aggressive disease, with a rapid need for treatment[103,106]. Recognizing this heterogeneity, Rai *et al.*[107] and Binet *et al.*[108] developed staging systems for assessment of the extent of disease in an individual patient. According the Binet and modified Rai[109] staging systems, the patient population falls into three prognostic subgroups, which is based on factors reflecting the overall tumor burden. In general, both clinical staging systems correlate well with prognosis. Rai staging has been applied mostly in North America, whereas Binet staging has been a preferred choice for clinicians in Europe[110]. In 1981, the International Working Group on CLL (IWCLL) has proposed the integration of both staging systems. However, the recommendation has not received widespread usage, and clinicians continue to use either system in both patient management and clinical trials[111].

Due to their simplicity and reproducibility, these systems have been widely applied, and their prognostic value has been validated in many studies. However, the staging systems do not accurately predict clinical outcome of CLL patients in the low risk subgroups who are likely to progress or not responding to therapy. Due to these reasons, several biological variables have been identified to refine prognosis and response to therapy in CLL patients.

### Mutation Status of IGHV genes

In 1999, two independent groups reported that CLL consisted of two subsets; the mutated and unmutated groups, which differ in prognosis and clinical course[32,33]. Importantly, CLL patients with mutated IGHV genes were shown to have good prognosis and lower chance of developing progressive disease, whereas those with unmutated IGHV genes have a shorter survival and a higher risk to succumb to progressive disease[32,33]. In one of the initial studies, the median survival of patients with mutated IGHV genes, defined as having less than 98% identity to germline sequences, was around 25 years, whereas the median survival of patients with unmutated IGHV gene was only 8 years[33]. The prognostic significance of IGHV mutation status has since been confirmed in several other independent cohorts[112-115], and is now considered to be one of the strongest prognostic markers in CLL.

**Genomic Aberrations**

Although there is no common genetic aberration that is characteristic and diagnostic of all CLL patients, certain recurrent cytogenetic abnormalities do exist. Most of these aberrations can appear during the course of the disease, whereas none can be considered an early transforming event of CLL. Using fluorescence *in situ* hybridization (FISH) analysis, more than 80% of CLL patients with one or more cytogenetic aberrations were successfully identified. The common recurrent chromosomal abnormalities include del(13)(q14), trisomy 12, del(11)(q23) and del(17)(p13), with varied frequency among different studies based on large patient cohorts[112,116,117].

The most common chromosomal abnormality, occurring in 40% to 60% of CLL cases, is del(13)(q14)[112]. CLL patients with del(13)(q14) as a sole genetic abnormality show a favorable prognosis[121]. Recently, it was shown that patients bearing homozygous del(13q), who mostly carried mutated IGHV genes, showed a tendency to better survival compared to patients with heterozygous del(13q)[118]. The exact target gene in the 13q-deleted region has remained unknown, but it was recently observed that both microRNA genes *miR-15a* and *miR-16-1*, which are located in the 13q14 region, are deleted or down-regulated in up to 70% of CLL cases[119,120]. Moreover, *miR-15a* and *miR-16-1* expression has been shown to inversely correlate to Bcl2 expression in CLL[121].

Trisomy 12 is the next most common abnormality, occurring in 15–20% of cases. It is associated with atypical morphology and an intermediate prognosis[122-124]. The 12q22 segment contains *CLLU1*, which is the first gene that was considered specific for CLL cells. High *CLLU1* mRNA expression has been associated with poor clinical outcome in young CLL patients[125]. However, no difference in *CLLU1* expression in patients with or without trisomy 12 has been reported[126].

del(11)(q23) occurs in 15% to 20% of CLL cases, and is associated with poor prognosis. Patients with 11q-deletion are generally younger and have more advanced Rai stages[112], and are more common among cases with unmutated IGHV genes, and ZAP-70 or CD38 positivity[123,127]. The region 11q22–23 contains the *ATM* gene[128]. This gene codes for a protein that acts upstream of p53 in the DNA damage response pathway. *ATM* gene mutations have been identified in CLL patients with 11q-deletion and are associated with an adverse outcome in CLL[129,130]. *ATM* mutations can be present in the germline of patients, suggesting that *ATM* heterozygotes may be predisposed to CLL[131].

del(17)(p13) occurs in about 5% to 7% of untreated cases, and it confers the worst prognosis among all the genetic abnormalities[112]. More than 70% of CLL patients with 17p-deletion harbor *TP53* mutation on the remaining allele[114,132] (described in other section). Other mechanism that deregulates p53 function, including those involving the *ATM* and murine double minute 2 (*MDM2*) genes, has also been observed in CLL[131,133].

### Therapy and Outcome

Therapy is not recommended for asymptomatic CLL patients with early disease ("watch and wait" approach), regardless of prognostic markers, as most of them will follow an indolent disease course. However, if the disease appears to progress, fludarabine in combination with cyclophosphamide will be the first treatment of choice. Chlorambucil is priorly given to the elderly or patients who cannot tolerate such treatment, with the aim to keep toxicities at minimum as well as keeping the patients symptom free[134]. Rituximab has been incorporated with fludarabine, and has shown better progression-free survival (PFS) and overall survival in previously untreated CLL patients[135]. Moreover, recent studies have demonstrated the effectiveness with therapy combining rituximab, fludarabine and cyclophosphamide, which has significantly improved the response rates[136-138] and PFS of CLL patients[136,139]. Alemtuzumab, an anti-CD52 monoclonal antibody has demonstrated efficacy in patients no longer responded to fludarabine, including 17p-deleted patients, and has shown impressive response when used as first line therapy[140]. Combination of rituximab and alemtuzumab has also been shown to improve the response rate of CLL patients with relapsed or refractory CLL[141].

## The p53 Pathway

The p53 pathway is composed of approximately 50 different enzymes regulating the p53 protein, and a network of genes and their products which respond to a different kinds of intrinsic and extrinsic stress signals[142]. Most of the positive and negative auto-regulatory feedback loops in the p53 pathway act through the Mdm2 protein[143]. Loss or mutation of the *TP53* gene is strongly associated with susceptibility to cancer, and most functions of this gene stem from its role as a tumor suppressor. Given the importance of p53 in responding to various cellular insults, p53 has been an almost universal target of somatic mutation in human tumors, including B-cell lymphomas. Apart from mutations, polymorphisms affecting the *TP53* locus and genes in the p53 pathway have also been reported.

## Structure and Function of p53

The p53 protein was identified in 1979[144,145] and its gene, which is called *TP53*, was cloned in 1983[146]. *TP53* is located on chromosome 17p13.1[146]. Its 393-residue protein can be divided structurally and functionally into five regions (Figure 4). The N-terminal region contains the transactivation domains (residues 1–42), followed by a proline-rich regions (residues 63–97). The transactivation domain interacts with several regulatory proteins, including the negative regulator Mdm2, which regulates cellular levels of p53 (described in the next subsection). The large core conserved region of p53 (residues 102–292) binds DNA with sequence specificity, often in the promoter area of target genes. p53 binds to its response elements with varying affinities depending on the sequence. Generally, p53 binds with high affinity to the recognition elements of genes involved in cell cycle arrest, whereas it binds with much lower affinity to genes involved in apoptosis[147]. The C-terminal region includes the tetramerization domain (residues 324–356), which regulates the oligomerization state of p53, as well as the negative auto-regulatory domain at the extreme C-terminus (residues 363–393), which contains acetylation sites and binds DNA without obvious specificity[148]. Three nuclear localization signals (NLS) that tag and target the p53 protein to the nucleus are also located in this area[149].

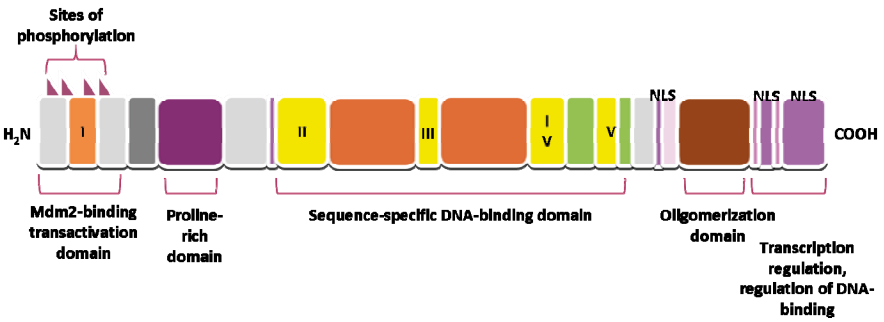


*Figure 4* Basic structure and function of the p53 protein.

p53 functions as a transcription factor that both positively and negatively regulates the expression of a large and distinct group of responsive genes[150]. Transactivation of target genes under the influence of p53 is an important feature of each stress response pathway; however, some effects of p53 may be independent of transcription[151]. The transcriptional activity of p53, in response to DNA damage and other cellular stresses, can lead to cell cycle arrest, apoptosis, and cellular senescence[152]. However, it is now evident that the ability of p53 to influence gene expression has reached broader effects,

including the regulation of glycolysis[153], autophagy[154], and the repair of genotoxic damage[155].

The importance of p53 function for tumor suppression is demonstrated by the high incidence and early onset of cancer with a genetic deletion or germ line mutation of *TP53*[156], in particular the mutation detected in patients with Li-Fraumeni syndrome (see below). *TP53* inactivation, which is often achieved through mutations affecting the *TP53* locus directly[157], occurs in over 50% of solid tumors[158,159]. Indirect inactivation through excessive activity of its major negative regulator, the Mdm2 protein has also been implicated in tumorigenesis[160]. Moreover, several single nucleotide polymorphisms (SNPs) have been identified within the *TP53* gene, including those affecting codons 72 (described in another section), 217[161] and 360[161], as well as in genes in the p53 pathway, which include the *MDM2* (described in another section), *ATM*[162] and *p21*[163] genes. Significantly, SNPs in these important genes of the p53 pathway have been demonstrated to modify function and/or may influence individual's susceptibility to cancer and progression of their disease.

## Mdm2: The Core Control of p53

The significance of Mdm2 as the main control of p53 is highlighted by the fact that amplifications of *MDM2* occur in about 5% to 10% of cancers, representing an alternative mechanism to inactivate p53 in the absence of mutation[164]. A polymorphism in the *MDM2* promoter (SNP309) leads to increased Mdm2 expression and accelerates tumorigenesis in individuals with germline *TP53* mutations as well as in some sporadic cancer cases[165] (see section below). Moreover, genetic inactivation of *MDM2* in mice is embryonically lethal but is rescued by inactivation of *TP53*[166].

The cellular levels of p53 is mainly achieved through an auto-regulatory negative feedback loop with Mdm2[167]. The *MDM2* gene has two promoter-enhancer regions that regulate the levels of *MDM2* mRNA. The first promoter is located 5' to the first exon and functions in regulation of the basal level of Mdm2 in an unstressed cell. The second promoter region is in the first intron and this region is responsible for the increased expression of Mdm2 after a p53 response[168].

In normal, unstressed cells, Mdm2 is an unstable RING domain that confers E3 ubiquitin ligase activity towards lysines in the C-terminus of p53[169], and induces proteosome-mediated degradation of p53. Mdm2 also exports p53 out of the nucleus, promoting its degradation and making it inaccessible to the target genes[170]. Most p53 are targeted for proteolysis; however, some

escapes the Mdm-mediated degradation to keep the feedback loop active. The p53 that escapes binds to a response element in *MDM2* intron 2 and transactivates *MDM2*, generating a feedback loop by which p53 controls its own degradation and return to basal levels after stress response[171]. Akt kinase has also been implicated in regulating Mdm2-mediated degradation of p53. Entry of Mdm2 into the nucleus is dependent on its phosphorylation by the phosphatidylinositol 3-kinase (PI3K)/Akt kinases, and activation of Akt by growth factors is sufficient to promote nuclear entry of Mdm2[172]. Upon phosphorylation, Mdm2 translocates from the cytoplasm to the nucleus where it ubiquitinates p53 and mediates its degradation.

Upon DNA damage (or ionizing radiation), Mdm2 binding to p53 is impaired, which leads to rapid stabilization of p53 through a block of its degradation[173,174] (Figure 5). The *MDM2* gene product is also transcriptionally incompetent due to various post-translational modifications (such as phosphorylation) of p53, Mdm2 or both proteins[152,175]. Phosphorylation of p53 by ATM makes p53 more resistant to inhibition by Mdm2 and increases its transcriptional activity[176], whereas phosphorylation of Mdm2 by ATM mainly impairs the ability of Mdm2 to promote p53 degradation[177]. On the other hand, activated oncogenes induce the ARF protein, which is a direct inhibitor of the E3 activity of Mdm2[178]. The ARF protein binds Mdm2 and inhibits the nuclear export of Mdm2 by sequestering it into the nucleolus[179]. The inhibition of p53 degradation will lead to activation of the protein. Consequently, the p53-dependent genes required for cell cycle arrest (e.g. *p21*) will be up-regulated and/or apoptosis will be induced[180]. After DNA is repaired, p53 and Mdm2 will receive a signal which results in modification of the proteins that allow them to form a complex. Once the complex is formed, p53 levels will be reduced, and this will stop the transactivation of p53-responsive genes. The cells will then resume progression through the cell cycle.

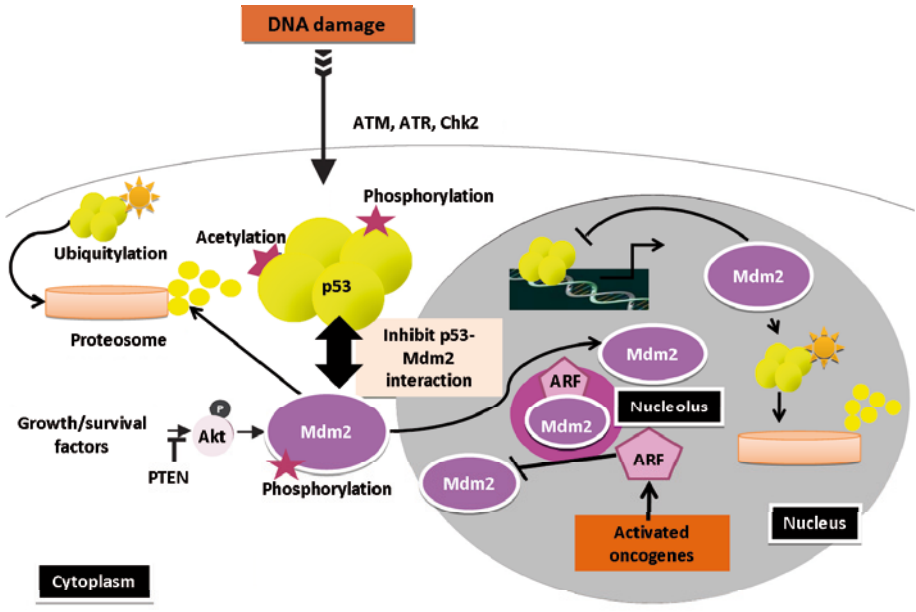


*Figure 5* The complex regulation of p53, in which Mdm2 appears to be the most important regulator of this protein. p53 and Mdm2 regulate each other through an auto-regulatory feedback loop. p53 transcribes the *MDM2* gene, and the Mdm2 protein, in turn, inhibits p53 transcriptional activity. Mdm2 promotes proteosome-mediated degradation of p53 through ubiquitination (or ubiquitylation), which can occur either by transporting it out from the nucleus, or in the nucleus itself. Mdm2 entry into the nucleus also depends on Akt activation. Upon DNA damage, ATM and ATR will send signals to CHK2 to phosphorylate p53, which results in impaired p53-Mdm2 binding and activation of p53. Phosphorylation of Mdm2 by ATM will disrupt its ability to promote p53 degradation, thus stabilizing p53. On the other hand, Mdm2 function can be inhibited by ARF. ARF, which is activated by oncogenes, binds to Mdm2 and sequesters it into the nucleolus, disabling Mdm2 to promote p53 degradation. Modified from Moll and Petrenko[181].

# Significance of the p53 Pathway in DLBCL and CLL

## Mutations of the *TP53* Gene

*TP53* mutation was first described in a group of Li-Fraumeni familial cancer syndrome patients[182]. The significance of *TP53* mutation has since been recognized in other cancers and lymphoid malignancies, including B-cell lymphomas. Most of these *TP53* mutations occur in exons 5 to 8, the coding region for the DNA-binding domain of the protein[159]. More than 80% of all mutations in *TP53* are single base substitutions in the DNA-binding domain[158], where changes in the amino acid encoded interrupt the interaction of

p53 with their target DNA sequences. The most frequent types of *TP53* mutations in lymphomas are missense mutations, followed by deletions and insertions, nonsense, silent and splicing mutations[183]. *TP53* missense mutations, particularly in the DNA-binding domain, lead to the formation of a stable mutant p53 protein. This mutant protein has lost its tumor suppressor property, and is incapable of binding to p53 target DNA in a sequence-specific manner[184]. However, some mutant p53 proteins are still capable of inactivating their target genes[184]. Among the frequently mutated residues, six hotspot codons were identified; codons 175, 245, 248, 249, 273 and 282[185]. *TP53* intronic mutations, which are usually detected within splice sites, could interrupt the normal splicing of introns[186].

As p53 is a coordinator of the cellular response to stress, its inactivation leads to development of more aggressive tumors. Patients with *TP53*-mutated aggressive tumors are associated to worse prognosis, increase rate of relapse and shorter survival[187-189]. *TP53* missense mutations often lead to nuclear accumulation of mutant p53 proteins, which might have unfavorable consequences due to the fact that some p53 mutant proteins exert the gain-of-function activity[190]. This acquired function is achieved through activation of a particular transcription program, or through dominant negative interaction with wild-type p53 on the remaining allele[190], or other proteins[184,191]. This confers tumorigenic potential by enhancing tumor cell growth or resistance to drug-induced apoptosis, thereby decreasing patient survival[192,193].

*TP53* mutations are detected at variable frequencies in hematologic malignancies, depending on the cell of origin and tumor type[194]. Mutations are found in 10% to 20% of B-cell lymphomas and have generally been associated with unfavorable outcome[194,195]. In DLBCL, mutations and deletions of *TP53* were detected in 10% to 23% of patients, where poor overall survival of these *TP53*-mutated DLBCL patients was indicated[46,187-189,196-198]. However, the impact of *TP53* mutation on patients outcome has not been consistently observed[199,200]. Recently, the negative prognostic impact of *TP53* mutation was reinforced in DLBCL patients[201,202]. Young *et al.*[202] reported that 24 of 113 (21%) *de novo* DLBCL cases displayed *TP53* point mutations (as well as a deletion and an insertion) in exons 5 to 8, where the presence of these mutations correlated with clinical parameters. The study was further conducted to analyze the relationship between structural profiles of *TP53* mutations and survival in DLBCL, and their relationship to gene expression profiles of different subtypes of DLBCL[201]. Importantly, a majority of *TP53* mutations were found in codons involved in DNA binding motifs of the central core domain of *TP53*, which was significantly associated with worse overall survival, compared to mutations occurring outside of this region[202]. It should be noted, however, that the correlation between p53 ex-

pression and *TP53* mutation in DLBCL has generally been less consistent than that of low grade lymphomas[203,204].

In CLL, genomic aberrations that modify the p53 expression, either by deletion or mutation, are often associated with aggressive disease and are independent poor prognostic factors[205]. Patients with 17p-deletions display poor survival, with short time to first treatment and high risk of chemorefractoriness to alkylating agents and purine analogs[112,114,124,132]. As mentioned, the recurrent 17p-deletion is detected in 5% to 7% of CLL patients in early stage disease, and is more common among patients carrying other poor prognostic factors[112,123,127]. Apart from 17p-deletion, *TP53* is also a target of somatic mutation in CLL. A majority (≥70%) of CLL patients harboring 17p-deletions also has *TP53* mutations[206-209], where 3% to 5% of CLL patients carry *TP53* mutations without 17p-deletion, and an even higher incidence (up to 18%) is observed in patients with fludarabine-refractory disease[206]. The negative prognostic impact of *TP53* mutation in the absence of 17p-deletion was initially documented by Zenz *et al.*[209]. The presence of *TP53* mutation was also demonstrated as an independent predictor of poor survival[208,209] and rapid disease progression[206]. Moreover, the survival and treatment response for patients with both 17p-deletion and *TP53* mutation, *TP53* mutation only, and 17p-deletion only were found to be equally inferior[208,209]. That notwithstanding, a subset of cases with 17p-deletion pursues a benign clinical course. This was documented by Best *et al*.[210], who showed that a subset of Binet stage A patients with 17p-deletion and mutated IGHV genes have a stable disease for several years without requiring therapy.

## *TP53* Codon 72 Polymorphism and *MDM2* SNP309

The study of *TP53* polymorphisms has increasingly attracted attention with the introduction of high-throughput genomic technologies using large cohorts. Over 3.1 million sequence variations have been mapped, which represents 25% to 35% of the total estimated SNPs[211]. Numerous SNPs and other sequence variations have also been identified at the *TP53* locus[212]. However, most are predicted to have no biological effects. It will be a challenge in the future to elucidate the potential cancer risk association for particular SNPs, as the effects of a polymorphism can be understated and can vary according to genetic background.

The first *TP53* exonic SNP discovered was codon 72 polymorphism (rs1042522) which involves a G→C substitution at nucleotide 466 of exon 4, resulting in either an arginine (CGC) or a proline (CCC) residue[213]. It is a non-synonymous SNP, and this polymorphism creates a p53 protein with reduced potential to induce apoptosis or suppress cell transformation[214]. The

polymorphic variants of codon 72 have been shown to have some differences in biochemical properties, with the arginine (Arg) allele exhibiting higher apoptosis-inducing activity, whereas the proline (Pro) allele appears to induce higher levels of the $G_1$ cell cycle arrest[215,216]. Moreover, the Pro/Pro phenotype demonstrates enhanced growth arrest and is a stronger inducer of transcription[214-216]. The *TP53* codon 72 polymorphism has been associated with poor prognosis and increased cancer risk in solid tumors such as ovarian and peritoneal carcinomas, breast cancers, and lung cancers[217-219]. However, its impact on hematologic malignancies needs to be further explored. So far, there has been no association of this polymorphism with patients' outcome in DLBCL[220] or CLL[221-223].

Many cellular proteins interact with or are under the control of p53. Polymorphisms in any of these proteins might influence cancer risk or synergize with *TP53* polymorphism and/or mutation to modify the risk. Several genes in the p53 pathway contain polymorphisms which might be potentially of interest in the clinical settings. One of these genes is the most well-characterized *MDM2*, bearing the polymorphism *MDM2* SNP309, as discussed below.

*MDM2* SNP309 (rs2279744) characterizes a T→G substitution at nucleotide 309 of intron 1 of chromosome 12[160]. This polymorphism localizes near the p53 response element and creates a higher-affinity DNA binding site for the transcription factor Sp1, resulting in increment in *MDM2* mRNA and protein in cells[160]. Cell lines homozygous for the G allele express high levels of Mdm2 and were shown to have an impaired p53 response after DNA damage, with poorly-induced p53 transcriptional activity and decreased p53-induced apoptosis[160]. The estrogen receptor also binds the *MDM2* promoter in the region of SNP309. In estrogen-responsive cells, estrogen preferentially induced the transcription of Mdm2 from the SNP309 promoter and the levels of Mdm2 in cells with homozygous G alleles were higher than cells with heterozygous T/G alleles or homozygous T alleles[224]. As a result, individuals carrying the homozygous G alleles might have higher levels of Mdm2 than individuals carrying the homozygous T alleles when estrogen levels are higher. The increased level of Mdm2 would lead to suppression of p53 function and might lead to a higher risk of hormone-related cancers. In DLBCL, a study by Bond *et al.*[225] have shown that homozygosity for the G allele was correlated with earlier onset of *de novo* DLBCL in women. Another study performed in a German DLBCL cohort has investigated the impact of SNP309 on age of DLBCL onset at diagnosis, as well as its impact on outcome[220]. However, no significant correlation with either disease onset or survival was observed. In CLL, the effect of *MDM2* SNP309 in prognosis of remains arguable due to conflicting results. This polymorphism has been

suggested as independent predictor of inferior outcome in CLL patients, with significant negative correlation found between the SNP309 T/G and G/G genotypes with overall survival[133]. The effect of the heterozygous SNP309 T/G genotype on treatment-free survival was found to depend on the p53 status but not on other known prognostic markers in CLL. However, a subsequent larger study found no association of this polymorphism to age of disease onset, survival or outcome, thus arguing the role of *MDM2* SNP309 as a prognostic factor in CLL[226].

## p16$^{INK4a}$

The *p16$^{INK4a}$* gene is one of the most frequently altered loci in human tumors besides *TP53*. Genetic or epigenetic inactivation of *p16$^{INK4a}$* has been observed in a proportion of B-cell lymphomas; it is commonly detected in aggressive (DLBCL, Burkitt's lymphoma) compared to indolent (FL, MZL, MCL, CLL) B-cell lymphomas. The predominant types of *p16$^{INK4a}$* inactivation include homozygous deletion or loss of heterozygosity, and hypermethylation of the *p16$^{INK4a}$* promoter.

### Structure of p16$^{INK4a}$

The p16$^{INK4a}$ protein was first identified in 1993 in coimmunoprecipitation experiments[227], and was later demonstrated to be a specific inhibitor of the cyclin-dependent kinase (CDK) 4/cyclin D kinase[228]. The *p16$^{INK4a}$* gene was independently isolated as a candidate tumor suppressor gene located at chromosome 9p21[229,230]. The locus encoding *p16$^{INK4a}$*, named *INK4a*, has the capacity to give rise to two distinct transcripts from different promoters[231] (Figure 6). Each transcript has a specific 5' exon, namely exon 1α and exon 1β, which share the common exons, exon 2 and 3. One promoter produces a transcript that is formed by exons 1α, 2 and 3 and encodes *p16$^{INK4a}$*, whereas the other promoter produces a transcript that is formed by exons 1β, 2 and 3 and encodes *p19$^{ARF}$* [232].

### Involvement of the *INK4a* locus in Rb and p53 Pathways

*INK4a* is the link between two major pathways that control cell cycle progression: the retinoblastoma (Rb) and the p53 pathways (Figure 6). The Rb pathway, which regulates cell cycle entry and progression through the $G_1$ restriction point, involves p16$^{INK4a}$, Rb, CDK4 kinase and cyclin D. The p16$^{INK4a}$ protein inhibits the activity of the CDK4-CDK6-cyclin D1 complex[233]. This latter complex increases the phosphorylation state of the Rb protein, causing it to release the transcription factor E2F. E2F binds to the

promoters of target genes, and mediates the transcription of several genes involved in the $G_1$ to S and $G_0$ to S transitions in the cell cycle[231]. Particular components of the Rb pathway are frequently altered in human cancers, including B-cell malignancies. This inactivation can be achieved by an increased activity of CDK-cyclin complexes and/or by inactivation of the tumor suppressor genes *p16*$^{INK4a}$ or *RB*[234]. The p53 pathway involves *p14*$^{ARF}$ (the human homologue of *p19*$^{ARF}$)[231], which acts together with *TP53*, *p21* and *MDM2*. The p53 pathway regulates the $G_1$ and $G_2$ checkpoints, or induces apoptosis in response to DNA damage or other cellular stresses, as previously described in this thesis.

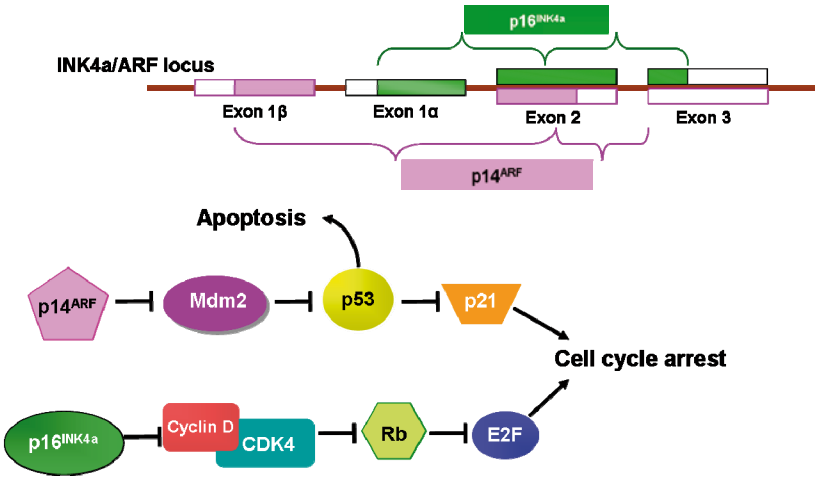


*Figure 6* The INK4a/ARF locus and the pathways involved. Exons 1α and 1β encode *p16*$^{INK4a}$ and *p14*$^{ARF}$, respectively. Exons 2 and 3 are common exons shared between the two genes. Loss or mutation of *p16*$^{INK4a}$ leads to inactivation of the Rb pathway (p16$^{INK4a}$, Cyclin D-CDK4, Rb), whereas inactivation of *p14*$^{ARF}$ leads to abrogation of the p53 pathway (p14$^{ARF}$, Mdm2, p53, p21). Thus, alteration of this single locus is highly efficient in compromising the two cancer pathways.

## *p16*$^{INK4a}$ Inactivation

The involvement of *p16*$^{INK4a}$ in the development of human tumors was implied by the observation that the *INK4a* locus was mutated in many tumor-derived cell lines and maps to a chromosomal region frequently altered in human malignancies[229,230]. Several mechanisms of *p16*$^{INK4a}$ inactivation have since been characterized, including deletion, mutation and hypermethylation, the latter which will be discussed further in the next subsection of this thesis.

*p16*$^{INK4a}$ is frequently inactivated by two deletion events, one on each homolog[235]. The high frequency of *p16*$^{INK4a}$ deletions in many types of tumors

suggested that this gene is a major tumor suppressor gene[236]. This finding has been reinforced by the analysis of knockout mice lacking functional *p16*$^{INK4a}$; these mice were found to spontaneously develop B-cell lymphoma[233]. In the initial reports, *p16*$^{INK4a}$ was shown to be homozygously deleted at high frequency in cell lines derived from a wide range of tumor types. It was then proposed that homozygous deletion is the predominant mechanism for inactivating *p16*$^{INK4a}$ in most tumor cell lines, including leukemia-lymphoma cell lines[51,229,230]. Point mutations of *p16*$^{INK4a}$ were rare in leukemias and lymphomas[229,230].

Previous studies have identified hypermethylation of the promoter of *p16*$^{INK4a}$ as an important mechanism of gene silencing[50,237,238]. Hypermethylation of a 5' CpG island might represent an alternative mechanism of inactivation of *p16*$^{INK4a}$ (see below). Although mutation has been a well-established factor leading to loss of tumor suppressor function, promoter hypermethylation of several well-known tumor-related genes may also effectively lead to the loss of gene function[239-241]. This epigenetic process may participate with genetic alterations to cause deregulation in gene function and expression, and may confer an additional selective advantage to tumors[241]. The mode of gene inactivation by DNA methylation is further discussed in the next section.

# Epigenetics and Gene Silencing by DNA Methylation

## Introduction to Epigenetics

The term epigenetics was first introduced by a British embryologist and geneticist, Conrad Hal Waddington in 1939, and it was used to describe "the causal interactions between genes and their products, which bring the phenotype into being"[242]. Nowadays, epigenetics is defined as the reversible and heritable stable changes based on gene expression levels, as opposed to genetics, which refers to information transmitted based on a change in the gene sequence[243]. Epigenetic modification has an important role in defining the transcriptome, which will eventually determine the characteristic of each cell type[244].

## DNA Methylation

The most widely studied epigenetic modification in humans is DNA methylation[245]. Besides global hypomethylation of DNA, which is the initial finding in human tumors[246], and hypermethylation of the promoters of tumor-suppressor genes[237,247], miRNA has emerged as one of the recent participant in epigenetic mechanism[248]. Today, the list of genes undergoing methylation

and silencing in different cancers continues to grow. In fact, methylation of particular genes can eventually affect all important cellular pathways in relation to cancer[245].

DNA methylation involves the transfer of a methyl group to cytosine that precede guanines, or CpG dinucleotides[249]. This enzymatic reaction is catalyzed through DNA methyltransferases and uses S-adenosyl-methionine as a methyl group donor. In general, over 85% of CpG dinucleotides are scattered in the human genome. The remaining 15% are clustered to form CpG island[250] which is typically more than 500bp in length, and frequently spans the 5′ end of the regulatory region (promoter, untranslated region and exon 1) of many genes[251]. CpG islands are normally unmethylated[252].

DNA methylation is important for normal development, chromosome stability, and maintenance of gene expression states and proper telomere length[253-255]. DNA methylation at CpG islands can directly inhibit the binding of transcription factors to their target sites, thus preventing the transcription of specific genes. Moreover, DNA methylation normally promotes a highly condensed heterochromatin structure, where active transcription does not occur, through recruitment of DNA-organizing proteins. As it is stable and heritable throughout cell divisions, DNA methylation allows the daughter cells to retain similar expression pattern as the parent cells, which is important for inactivation of the X chromosome and imprinting.

## Aberrant Methylation Pattern in Hematological Malignancies

DNA hypermethylation is a common mechanism for tumor suppressor gene inactivation in hematologic malignancies, including leukemias and lymphomas[256-258]. Most of the genes involved are associated with transcriptional silencing[249]. In B-cell lymphomas, the genes most frequently affected by hypermethylation-associated gene silencing include *MGMT*[239], *p57*[259] and *p16*$^{INK4a}$ [260] (see below), *DAPK1*[261] and *ZAP-70*[262]. Moreover, genome-wide DNA methylation analysis revealed several candidate genes significantly hypermethylated in B-cell lymphomas, including *DBC1*, *MYOD1*, *VHL* and *ABI3*[263,264].

The methylation status of *p16*$^{INK4a}$ has been studied in different types of lymphomas, including multiple myeloma[265], MCL[266], Burkitt's lymphoma[267], anaplastic large cell lymphoma[268] and MALT lymphoma[269]. It has been associated with aggressive transformation from indolent lymphoma subtypes[270,271]. p16$^{INK4a}$ loss of expression has also been related to tumor progression in several types of B-cell lymphomas[267,271,272]. On the other hand, *p16*$^{INK4a}$ methylation has been proposed to represent an early event related to

lymphoma onset[268]. Subsequently, hypermethylation of CpG islands in *p16*$^{INK4a}$ has been investigated as a prognostic factor in B-cell lymphomas, including DLBCL.

## Significance of *p16*$^{INK4a}$ Hypermethylation in DLBCL

In the past few years, promoter hypermethylation of *p16*$^{INK4a}$ has emerged as a contributing factor to the pathogenesis and progression of DLBCL. Despite the small number of DLBCL cases analyzed (between 9–68 cases in each study), *p16*$^{INK4a}$ methylation has been observed in 16% to 54% of cases[198,260,268,270,273-275]. However, the prognostic impact of *p16*$^{INK4a}$ methylation in this disease remains unclear, although it has been suggested to associate with the clinical course[260,268,270,273]. Shiozawa *et al.*[260] reported that hypermethylation of *p16*$^{INK4a}$ significantly correlated with an inferior overall survival in patients with intermediate–high IPI groups. On the contrary, Amara *et al.*[273] showed that DLBCL patients with hypermethylated *p16*$^{INK4a}$, particularly in the low–intermediate risk groups, had poorer outcome. In addition, Gronbaek *et al.*[274] and Sanchez-Beato *et al.*[198] have observed the negative prognostic impact of *p16*$^{INK4a}$ methylation in patients with concurrent alterations of other genes (such as *TP53*, *p14*$^{ARF}$ and *p27*). DLBCL patients with concurrent *TP53* mutation, *INK4a/ARF* locus deletions and *p16*$^{INK4a}$ methylation were shown to have a significantly worse prognosis than those with no concomitant alterations of the *ARF* and *TP53* genes[274]. The survival rate of patients with alterations in *TP53*, *p16*$^{INK4a}$ and *p27* was also significantly lower compared to patients with *TP53* mutations alone or no alterations[198].

## Approaches for Gene-specific DNA Methylation Analysis

### Sodium bisulfite modification of DNA

A variety of methods have been developed to assess the methylation status of specific genes qualitatively, as well as quantitatively. Most methods rely on the sodium bisulfite modification of genomic DNA prior to polymerase chain reaction (PCR) amplification, which has been paired with several techniques other than traditional sequencing. These include the methylation-specific PCR (MSP)[276] (see below), bisulfite genomic sequencing[277], pyrosequencing[278] (see below), and methylation-sensitive high-resolution melting-curve (MS-HRM) analysis[279]. Under successive bisulfite treatment, all unmethylated cytosines will be converted to uracil, whereas the methylated

cytosines (5-methylcytosines) remain unchanged. Subsequently, uracils will be replicated as thymine in the PCR amplification.

The integrity of bisulfite modification of DNA can be challenged by inappropriate conversion of 5-methylcytosine to thymine, and unsuccessful conversion of unmethylated cytosine to uracil, the latter being a more common example of bisulfite-conversion error[280]. Hence, it is important to ensure adequate and complete bisulfite treatment by assessing the bisulfite conversion quality.

## Methylation Detection by MSP

MSP is a rapid and cost-effective method to screen for DNA methylation of known genes in a relatively large number of samples. As it requires no specialized equipment other than those available in a routine laboratory, MSP has been widely accepted as a method to analyze methylation at a specific locus. MSP was initially developed to assess promoter methylation status at CpG islands in cell lines and clinical materials, including fresh or frozen tissues[276]. This method distinguishes between methylated and unmethylated bisulfite-converted DNA by utilizing two pairs of specific amplification primers in separate PCR reactions.

The major advantage of MSP is its high sensitivity, being able to detect one methylated allele in a population of more than 1000 unmethylated alleles at a given CpG island locus[276]. Moreover, this method can be performed on limited quantity of DNA, as well as on DNA extracted from paraffin-embedded samples, as long as there is no excessive degradation of the DNA itself after bisulfite conversion. However, its major advantage is also a major drawback; its highly-sensitive nature makes MSP very susceptible to false-positive results[281-283]. An example of this is the incorrect interpretation of the unconverted unmethylated cytosines, which results from incomplete bisulfite-conversion of the DNA template, as methylated cytosines by the MSP primers. Moreover, this technique cannot distinguish tumors with a low or high proportion of methylated tumor cells. As the information obtained by MSP is qualitative, other methods, such as pyrosequencing[278], can be applied for quantitative assessment of methylation. The pyrosequencing technique is further described in the next section.

## Quantification of DNA Methylation by Pyrosequencing

Pyrosequencing is a real-time DNA sequencing method which, like most other methods for DNA methylation analysis, relies on bisulfite-conversion[278]. After bisulfite modification of DNA, the region of interest is

PCR-amplified, with either one of the two PCR primers biotinylated. The labeling is used to generate a single-stranded template to which the pyrosequencing primer will anneal.

Pyrosequencing is based on the transformation of pyrophosphate ($PP_i$) into measurable light, which is proportional to the number of incorporated nucleotides[284] (Figure 7). $PP_i$ is released upon incorporation of the nucleotide(s) by the Klenow fragment of the DNA polymerase I from *Escherichia coli*. The $PP_i$ is rapidly converted into ATP by the enzyme ATP sulfurylase, which provides the energy for another enzyme, luciferase to generate light. This reaction is accomplished at a very high speed and the light produced can be registered with a charge-coupled device camera (CCD), enabling a quantitative measurement of the incorporated nucleotides[285]. Before the addition of the next nucleotide, unincorporated nucleotides will be degraded by the enzyme apyrase. Pyrosequencing has been optimized so that only one nucleotide is present at any time in the reaction mixture.

Pyrosequencing assay is robust and the results are highly reproducible. It can be applied for DNA methylation analysis of CpG-rich regions as well as CpG-poor regions, where the quantitative evaluation of the methylated CpG sites is not achievable using a qualitative method, such as MSP. An improvement to the conventional pyrosequencing (i.e. serial pyrosequencing) has enabled the amplification of up to 300bp of the region of interest using several sequencing primers which annealed on the same template[286]. This is crucial in cases where precious clinical samples are analyzed, where unnecessary additional PCR amplification could be avoided.

However, there is one main limitation with this technique. Pyrosequencing is not meant to be used for genomic sequencing due to the limitation in the reading length, which is restricted to 350bp or less[285,287]. Amplification of more than the suggested reading length should be avoided, as secondary structure such as loops may form in the single-stranded template, which could eventually interrupt with the sequencing reaction or increase the background (noise) signal due to the extension of the 3'-end terminus.

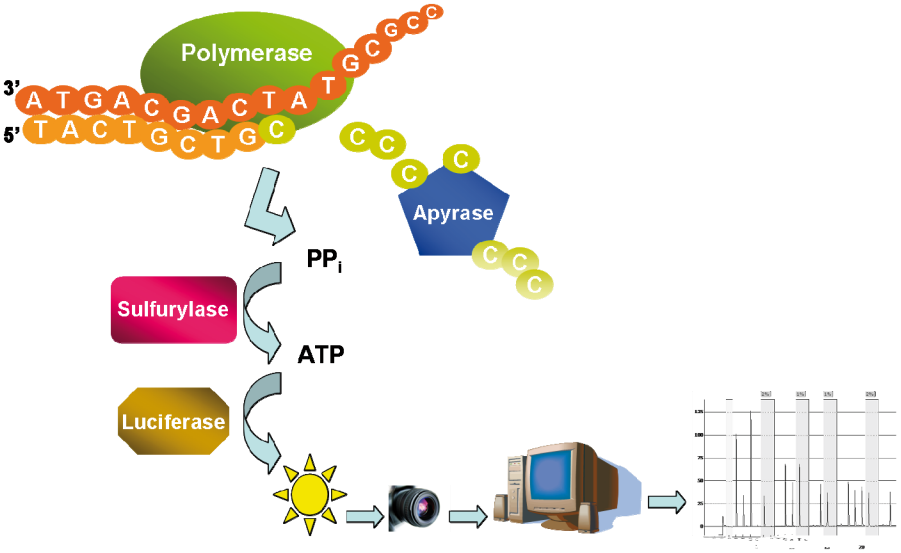


*Figure 7* Schematic representation of the pyrosequencing technique. The sequencing primer is hybridized to a single-stranded DNA template. The complementary strand is then synthesized in the presence of enzymes and substrates. The pyrosequencing process is initiated by addition of dNTP, one at a time. The incorporation of dNTP to the complementary DNA strand, which is catalyzed by the enzyme DNA polymerase, is accompanied by the release of $PP_i$. $PP_i$ is then converted to ATP by the enzyme ATP sulfurylase. The light signal, which is produced in presence of ATP and catalyzed by the enzyme luciferase, is detected by a CCD camera. The resulting peaks correspond to the number of incorporated nucleotides, which can be seen in the Pyrogram. Excess ATP and unincorporated nucleotides will be degraded by the enzyme apyrase.

# AIMS

Overall, the main objective of this thesis was to investigate the impact of *p53* and its pathway, as well as $p16^{INK4a}$ on the clinical outcome of DLBCL and/or CLL patients. Specifically, the work in this thesis aimed to accomplish the following objectives:

I To investigate the clinical relevance of *TP53* mutations, the *MDM2* SNP309 and the *TP53* codon 72 polymorphism in *de novo* DLBCL, and determine the impact of these aberrations on survival, particularly in GC and non-GC DLBCL.

II To analyze the extent of CpG hypermethylation of the $p16^{INK4a}$ promoter in *de novo* DLBCL patients quantitatively using the pyrosequencing technique, as well as qualitatively using MSP, and to correlate $p16^{INK4a}$ methylation status with clinical features and disease outcome.

III To investigate the prognostic role of *MDM2* SNP309 in CLL patients, and to correlate the genotypic data with well-established prognostic markers.

IV To determine the presence of *TP53* mutations in CLL patients at diagnosis from a population-based cohort of patients, and to determine if the frequency of *TP53* mutations is comparable to those reported in earlier studies.

# MATERIALS AND METHODS

## Patients and Tumor Specimens

All patients included in the studies were diagnosed according to the criteria in the WHO classification for each disease. The study population consisted of 102 and 113 *de novo* DLBCL (Papers I and II, respectively), and 268 and 418 CLL patients (Paper III and IV, respectively). All studies have been ethically approved by the local ethical review committee. Clinical information was available for all patients. In Paper I, DLBCL specimens were obtained between 1984 and 2002 from 102 patients (median age 66 years, range, 15–90), whereas the tumor material in Paper II were collected between 1984 until present from 113 DLBCL patients (median age 66 years, range, 15–90). In Paper III, CLL samples were collected from 418 Swedish CLL patients (median age 64 years, range, 32-89). Tumor samples from 169 of these cases were collected between years 1981 and 2006; the remaining 249 cases collected from the Swedish part of a population-based case-control study called SCALE (Scandinavian Lymphoma Etiology)[288] were obtained between the years 1999 to 2002. A total of 271 patients from the SCALE study were included in Paper IV. In Papers I and II, tumor materials were obtained from frozen DLBCL tumor biopsies, whereas in Papers III and IV, tumor materials were obtained mainly from the peripheral blood and bone marrow, with a smaller proportion obtained from lymph nodes and spleen. Genomic DNA was extracted using several different commercial kits, according to the manufacturers' protocols.

## *TP53* Mutation Analysis

In Papers I and IV, PCR amplification was performed using primers for exon 4, exons 5 to 6 and exons 7 to 8 of the *TP53* gene, followed by sequencing[289]. The Genome Assemble Program (GAP) software version 1.5 and the GenBank data library (release 160.0) were used for DNA sequence data analysis in Paper I, whereas the ContigExpress (Vector NTI Advance version 10.3.1, Invitrogen) and the GenBank data library (release 174.0) were applied to analyze and align sequences in Paper IV. BioEdit Sequence Alignment Editor Version 7.0.5.3 was also used for data analysis in both

papers. In Paper I, the performance of direct sequencing was validated using the U-2932 cell line, which harbors a known *TP53* mutation[290]. Mutated DNA from the cell line and normal DNA from a healthy control were mixed in different ratios ranging from 100% to 10%, and the cut-off for mutation detection was determined at 20%. Cases with negative PCR products were subjected for *TP53* deletion analysis using two informative microsatellite markers; p53CA, located upstream of *TP53* gene and D17S1678, located telomeric to the gene. To further confirm the results obtained from the deletion analysis, an independent PCR protocol using alternative set of p53 primers for each of exons 4 to 8 was carried out. Mutations were validated using the IARC *TP53* Mutation Database[291] and the UMD *TP53* Mutation Database[292].

## SNP Genotyping Analysis

In Papers I and III, *MDM2* SNP309 genotyping was performed using PCR-restriction fragment length polymorphism (RFLP)[293]. To differ between the *MDM2* genotypes, the resulting 154bp PCR products were digested with the *MspA1*I endonuclease and the digested products were then visualized on either a polyacrylamide gel (Paper I) or a 2% agarose gel (Paper III). The G/G homozygote product is cleaved by *MspA1*I and yields one 59- and one 95bp band, the T/T homozygote is not cleaved by the enzyme and thus yields a single 154bp band, whereas the T/G heterozygote contains all three bands. In Paper III, genomic DNA was subjected for whole-genome amplification (WGA) and the whole-genome amplified DNA was used for genotyping. Cross-confirmation of the genotyping findings was then performed on selected samples, where concordant results were achieved between the original genomic DNA and the corresponding whole-genome amplified DNA. The identification of codon 72 polymorphism in Paper I was carried out using the same PCR-RFLP technique. The resulting 312bp PCR products of *TP53* exon 4 were digested with *Bst*UI. The codon 72 wild-type Arg/Arg homozygote product is cleaved by this endonuclease and yields one 259- and one 53bp band, the Pro/Pro homozygote is not cleaved by the enzyme and yields a single 312bp band, while the Arg/Pro heterozygote contains all three bands.

## IGHV Mutation Analysis

In Paper III and IV, IGHV gene mutational status was investigated using PCR amplification with IGHV gene subgroup-specific primers and subsequent sequencing as previously described[294,295]. The sequences were aligned

to IG sequences from the Basic Local Alignment Search Tool (BLAST) database (National Center for Biotechnology Information, Bethesda, MD) and the international ImMunoGeneTics database IMGT/V-QUEST[296,297]. IGHV gene sequences with less than 98% identity to the corresponding germline gene were defined as mutated.

## Genomic Aberration Analysis

In Paper III, cytogenetic screening for recurrent genomic aberrations was performed using a commercial CLL FISH probe panel detecting del(17)(p13), +12, del(11)(q22) and del(13)(q14). An additional 130 samples were analyzed using high-resolution Affymetrix 250K SNP-arrays from which data on recurrent genomic aberrations were available[298]. All 268 CLL samples in Paper IV were analyzed using high-resolution Affymetrix 250K SNP-arrays.

## Pyrosequencing Assay

Genomic DNA was subjected to sodium bisulfite modification and subsequent PCR. Pyrosequencing assays initially developed at Biotage AB, Uppsala, Sweden were used to assess methylation status of the *p16*$^{INK4a}$ promoter (Ref: 40-0056). This assay detects the level of methylation in a region +148 to +182 in exon 1 of the *p16*$^{Ink4a}$ gene (Ensembl gene: ENSG00000147889). The degree of methylation of four CpG sites was analyzed by the PyroMark™ Q24 software. An internal control to check for adequacy of bisulfite treatment was included in each assay. The exact frequency of methylation could be determined by the resulting peak patterns, which differed between methylated and unmethylated samples. Methylation was quantified in terms of methylation level (MtL), which was the mean percentage of methylated cytosines per CpG ($\sum\%C^m/4$). Pyrosequencing can detect as low as 2.8% methylation level in totally (100%) unmethylated DNA, whereas a 91.7% methylation level could be detected in 100% *in vitro* methylated DNA.

## MSP Assay

In Paper II, two sets of primers were used for methylated and unmethylated *p16* promoter regions, and MSP assays were performed on bisulfite-converted DNA to determine the methylation status of *p16*$^{INK4a}$ promoter, as described previously[276], with minor modifications. This assay detects methylation in 18 CpG sites; four of which were included in the pyrosequencing

assay. Several steps were also performed to ensure the specificity of the PCR primers in the detection of methylated bisulfite converted or unmethylated bisulfite converted DNA. MSP results were scored when a clear visible band on the electrophoresis gel with the methylated and/or unmethylated primers was observed[276]. Methylation analysis was confirmed with repeat MSP assays after an independent bisulfite treatment in cases where faint bands were observed.

## Statistical Analysis

Statistica version 7.1 (Statsoft, Tulsa, OK, USA) was applied for all calculations in Papers I and III, Statistica version 8 was applied in Paper IV and Statistica version 9 was applied in Paper II. Fisher's exact tests and Chi Square analysis were employed to determine the significance of *TP53* mutation status (Paper I) and genotype usage (Paper I and III) between patient groups. Comparison of median age at diagnosis between different SNP genotypes was performed using Wilcoxon Mann-Whitney (Paper I) and Kruskal-Wallis tests (Papers I and III). The Kaplan-Meier analysis was carried out to estimate lymphoma-specific survival (LSS; Paper I), overall survival (Paper III), progression-free survival (PFS; Papers I and II) and time to treatment (Papers III and IV). Differences in survival and median time to treatment were evaluated using the log-rank test. Overall survival and LSS were calculated from date of diagnosis to the date of the last follow-up or death, the later being death caused by lymphoma disease. PFS was determined from the date of diagnosis to the date of occurrence of the first relapse. Time to treatment was evaluated by the time interval from date of diagnosis to date of the first treatment. Patients alive and in remission at last follow-up were censored from the analysis.

# RESULTS AND DISCUSSIONS

## Paper I – *TP53* Mutations, *TP53* codon 72 polymorphism and *MDM2* SNP309 in DLBCL

The impact of *TP53* mutations on survival in DLBCL remains controversial, with several studies showing associations with poor outcome[46,187,189,202], while other studies failed to demonstrate any significant correlation[199,200]. To better understand the clinical consequences of *TP53* mutations, we here investigated the prognostic impact of *TP53* mutations in a series of 102 patients with *de novo* DLBCL. We chose to investigate *TP53* mutations in exons 4 to 8 due to the findings that most human tumor mutations are located in this DNA-binding region of p53. In DLBCL, 10% to 23% of *TP53* mutations has been documented previously[187-189,196,197,202]. In our series of DLBCL patients, 12.7% of *TP53* mutations were detected, all of which were identical to common mutations reported in the IARC *TP53* Database[291] and Universal Mutation Database[299]. Most of the mutations identified in our series are missense mutations, with three cases showing mutations in the hotspot codons. *TP53* mutations were detected in 10.6% of GC DLBCL, whereas mutations were present in 14.5% of the non-GC subtype.

Overall, the *TP53* mutations identified in the present study predicted for poor LSS in DLBCL, with a greater prognostic value observed in the GC subtype (LSS, P=0.002). Our findings are thus comparable to previous studies in DLBCL which correlates the presence of *TP53* mutations to inferior outcome in patients[199,200]. Moreover, our results are in line with a recent study of *TP53* mutations in different subtypes of DLBCL[201], where they showed a tendency towards poor overall survival in *TP53*-mutated patients with the GC subtype. This finding was later confirmed in their subsequent larger study where *TP53* mutations were found to stratify patients prognostically within the GC subgroup[201]. In parallel with these studies[201,202], no significant difference was observed in survival among non-GC DLBCL patients. We suggest that the pronounced clinical effect of *TP53* mutations in GC DLBCL might be due to the involvement of NF-κB. NF-κB has been shown to suppress p53 transactivation and inhibit the transcription-dependent induction of apoptosis by p53[300]. Recently, it has been demonstrated that mutant p53 is able to increase the activity of NF-κB, which could

protect the tumor against chemotherapy-induced death[301,302]. We thus hypothesize that *TP53* mutation exerts its effect by enhancing the anti-apoptotic activity of NF-κB in GC-DLBCL, thus rendering a growth advantage in this tumor type. In contrast, mutant p53 may have a limited effect in non-GC DLBCL, since NF-κB is constitutively active in this subtype.

Since *TP53* mutations have been shown to be of relevance concerning patients' outcome, we therefore investigated the potential clinical relevance of the *TP53* codon 72 polymorphism in the same 102 DLBCL patients, with respect to survival. Previous studies have shown that the *TP53* codon 72 genotypes, particularly the homozygous Pro genotype, have a negative impact on survival in various human cancers[217-219,303]. Nevertheless, it has also been shown in several other studies that codon 72 genotypes were not clinically relevant for survival[221,223]. From the present study, we could not find any significant difference in LSS and PFS in relation to different codon 72 genotypes. Furthermore, no significant difference was observed in median age at diagnosis. We here conclude that different codon 72 genotypes do not appear to have an impact on prognosis and survival in DLBCL patients.

We further investigated the impact of another polymorphism in the *MDM2* gene: the *MDM2* SNP309. As observed in other studies correlating survival and presence of codon 72 polymorphism, conflicting results have also been reported regarding *MDM2* SNP309 and its association with survival and disease onset in various cancer types. Several studies have correlated SNP309 with poor survival[293,304-306]; other studies, however, failed to demonstrate any impact of SNP309 on age of onset[307] or survival[308]. No published data has correlated this polymorphism with DLBCL other than a study by Bond *et al.*[225] who found an accelerated age of diagnosis in female DLBCL patients carrying the G-allele. In the current study, we could not find any association between age at diagnosis and the different SNP309 genotypes, either in male or female, which contradicts the previous report by Bond *et al.*[225]. Moreover, no significant difference was found in LSS and PFS between patients with different SNP309 genotypes. Thus, *MDM2* SNP309 may not serve as a prognostic marker since it appears to lack clinical relevance in DLBCL.

## Paper II – $p16^{INK4a}$ Methylation in DLBCL

The $p16^{INK4a}$ gene was shown to be transcriptionally silenced by hypermethylation in a number of hematologic malignancies, including B-cell lymphomas[266,267,272]. Promoter hypermethylation of this gene has also been associated with transformation and tumor progression in lymphomas[270,271]. In

DLBCL, *p16*$^{INK4a}$ methylation has been reported to be more frequently detected in male patients[260], as well as in patients presenting clinical stage III-IV, higher IPI scores, a performance status of two and above, and B-symptoms[273]. *p16*$^{INK4a}$ methylation has been indicated to have a negative prognostic impact in DLBCL. However, this has remained unclear. Shiozawa *et al.*[260] has shown a correlation between *p16*$^{INK4a}$ hypermethylation and inferior outcome in intermediate–high risk IPI patients[260], whereas Amara *et al.*[273] has shown , in a later study, a correlation between *p16*$^{INK4a}$ methylation and poor overall survival in the low–intermediate risk IPI patients, but not in the higher IPI groups[273].

To further investigate the clinical significance of *p16*$^{INK4a}$ methylation in DLBCL, we have analyzed 113 DLBCL cases for the presence of *p16*$^{INK4a}$ methylation, and correlated it with patients' characteristics and survival. As previous *p16*$^{INK4a}$ methylation studies in DLBCL have utilized the qualitative MSP method to assess for the methylation status of this gene, we have taken a step further to quantify the degree of methylation in our DLBCL material. To the best of our knowledge, the present study is the first to apply the pyrosequencing technology to quantitatively assess *p16*$^{INK4a}$ methylation in DLBCL, and to correlate it with clinical characteristics and outcome.

In the present study, we have mainly investigated the significance of *p16*$^{INK4a}$ methylation status by comparing cases with unmethylated and cases in different categories of methylation (i.e. low, intermediate and high methylation). Based on the pyrosequencing data, cases with *p16*$^{INK4a}$ methylation level above 5% was interpreted as methylated, and this was observed in 42 (37.2%) of 113 DLBCL, which is in range of the previously reported *p16*$^{INK4a}$ methylation prevalence in DLBCL (16%–54%)[198,260,268,270,273-275]. Among the cases with more than 5% methylation level, 19 (45.2%) were in the low methylation category, 15 (35.7%) in the intermediate, and 9 (21.4%) in the high methylation category.

A qualitative methylation detection technique, MSP, was also employed in this study. Using pyrosequencing and MSP techniques, 79% of the cases showed concordant results for MSP-positive cases. However, more than half of MSP-negative cases also demonstrated *p16*$^{INK4a}$ methylation using pyrosequencing, although a lower level was detected in most cases. This could probably be attributed to the higher sensitivity of pyrosequencing in detecting methylation compared to MSP. Moreover, MSP is not able to distinguish between cases with a low or high proportion of methylated tumor cells, which is probably the case with heterogeneous tumors like DLBCL. In this regard, pyrosequencing thus represents a superior technique to determine the methylation status in clinical materials.

We have further evaluated the potential association of *p16*$^{INK4a}$ methylation with clinical characteristics. In contrast to previous reports, we found no correlation between the extent of *p16*$^{INK4a}$ methylation and any clinical variables, including age at diagnosis, IPI score and disease subtype, except a borderline significance for disease stage (P=0.049). Furthermore, despite applying a number of methylation cut-offs and inclusion criteria, we could not demonstrate the negative prognostic impact of *p16*$^{INK4a}$ methylation on patients LSS. Moreover, the negative prognostic impact of *p16*$^{INK4a}$ methylation on the survival of intermediate to high-risk IPI patients, as previously reported (13, 16), could not be verified in the present study. Nevertheless, in contrast to the inferior survival previously seen in patients with hypermethylated *p16*$^{INK4a}$, we observed an increase in PFS of young patients (<65 years of age) with methylation level above 25% compared to young patients with 25% or less methylation (P=0.048). The relative impact of *p16*$^{INK4a}$ methylation (MtL >25%) on PFS in these patients was further demonstrated in a multivariate analysis. Although it appears as if *p16*$^{INK4a}$ methylation with a methylation above 25% contribute to better outcome, this should be interpreted with caution, as only a proportion of selected DLBCL cases were analyzed. The relevance of this finding, if any, need to be further evaluated and validated. Hence, our findings question the role of *p16*$^{INK4a}$ promoter methylation as a negative prognostic factor in DLBCL.

## Paper III – *MDM2* SNP309 in CLL

The impact of *MDM2* SNP309 on survival of CLL patients remains controversial. The first reported study by Lahiri *et al.*[222] on 83 CLL patients failed to show any impact of the *MDM2* polymorphism on clinical outcome. Following this observation, a detailed analysis on *MDM2* SNP309 was performed by Gryshchenko *et al.*[133] in larger independent cohorts of 140 and 111 CLL patients to determine the prognostic role of this polymorphism. These investigators showed that *MDM2* SNP309 predicts for poor outcome, with significant correlation of the SNP309 TG and GG genotypes with overall survival. Moreover, patients carrying the GG genotype showed a more aggressive course of disease, with significantly reduced treatment-free survival, compared to patients with the TT genotype. Gryshchenko *et al.*[133] also demonstrated the independent prognostic role of SNP309 in both cohorts, where *MDM2* SNP309 predicted for treatment-free survival regardless of IGHV mutation status, CD38, ZAP-70 and Rai staging. A latter group, however, found no impact of SNP309 on disease course or outcome in their cohort of 617 CLL patients[226]. These conflicting reports on the role of *MDM2* SNP309 as a predictor of poor outcome of CLL patients has led us to further investigate this polymorphism in our cohort of 418 CLL cases. In addition,

we have also investigated the possible correlation of different *MDM2* SNP309 genotypes to well-established prognostic markers in CLL, such as IGHV mutation status, Binet stage and chromosomal aberrations.

Of all cases included in the present study, approximately 10% carried the homozygous G genotype, whereas the homozygous T and heterozygous TG genotypes were displayed in approximately 44% and 45% of the patients, respectively. There was no difference in median age at diagnosis between the different genotypes. The majority of the patients were classified into Binet stage A (72.5%), while the remaining were in Stage B (19%) and C (8.5%). However, no significant difference was observed when the different SNP309 genotypes were compared to different Binet stage subgroups. Furthermore, more than half of the CLL patients displayed mutated IGHV genes (58.4%); however, as observed within different Binet stages, none of the specific genotypes were significantly correlated with IGHV mutation status. Moreover, there was no significant association between the distributions of SNP309 genotypes in distinct cytogenetic subgroups, except for trisomy 12. However, the relevance of this observation, if any, is unknown. On the other hand, our data confirmed that unmutated IGHV genes, Binet stage B and C, as well as the presence of 17p-deletion, 11q-deletion and trisomy 12 can serve as poor-prognostic markers in CLL[33,112].

In line with the findings of Zenz *et al.*[226], we failed to observe any impact on specific SNP309 genotypes with overall survival and time to treatment in our cohort of CLL patients. Furthermore, we could not find any correlation between *MDM2* SNP309 with overall survival and time to treatment in relation to Binet stage, IGHV gene mutation status or chromosomal aberration subgroups. Thus, we conclude that *MDM2* SNP309 does not seem to have any impact on the clinical course of CLL. Unless confirmed in several subsequent larger studies, this polymorphism should not be considered as a new prognostic marker to predict clinical outcome in CLL.

## Paper IV – *TP53* Mutation in CLL

The significance of *TP53* mutation in the absence of 17p-deletion on survival in CLL was initially demonstrated by Zenz *et al.*[209]. *TP53* mutation without 17p-deletion, which was found in 4.5% of their CLL cohort, was associated with poor prognosis. Later the same year, Dicker *et al.*[206] showed that that isolated *TP53* mutation (4.7%) predicted rapid disease progression independently. Rossi *et al.*[208] and Malcikova *et al.*[207] could also confirm the negative prognostic impact of *TP53* mutation in the absence of 17p-deletion.

Most of these studies however consisted of patients from referral centers [206,208,209].

In this study, 268 newly diagnosed CLL patients from a population-based CLL cohort were analyzed for *TP53* mutations. Sixty-seven percent of patients in our cohort harbored mutated IGHV genes and the majority were in Binet stage A. Most of the patients in this study had 13q-deletions or no recurrent aberrations, whereas 17p-deletion was detected in 3.7% of cases. Based on these observations, the current cohort of CLL patients comprised a more prognostically favorable characteristic or "low-risk" patients than earlier studies on *TP53*[206-209].

Overall, we detected a low proportion (3.7%) of *TP53* mutations in the CLL patients included in this study. Most of these patients (7 of 10) presented with 17p-deletion on the remaining allele. Hence, a lower frequency of *TP53* mutations in the absence of 17p-deletions (1.1%) was observed in our cohort compared to other studies in CLL, which have shown frequencies ranging from 4%–5% of cases[206-209]. The lower overall incidence of *TP53* mutations without 17p-deletions in this study may reflect the inherent differences between this newly-diagnosed population-based CLL cohort compared to referred patient materials[206,208,209]. While the previous studies tend to have a selection bias towards more aggressive cohorts, our population-based cohort is comprised of more indolent cases, which is more representative of the *TP53* mutation prevalence in CLL at diagnosis.

The prognostic impact of 17p-deletion and *TP53* mutation in CLL has been previously reported[207-209]. In the present study, we have confirmed a significantly shorter overall survival ($P<0.0001$) and time to treatment ($P=0.01$) in patients with *TP53* mutations and 17p-deletions, compared to patients without any mutation or deletion. However, we could not properly analyze the impact of *TP53* mutation in the absence of 17p-deletion on patients' survival due to the low number of *TP53*-mutated patients in our material. Nonetheless, among the three patients with only *TP53* mutations, one had initiated treatment 5 months after diagnosis, but was deceased 66 months later; the second patient received treatment 24 months after diagnosis, and was alive at 95 months, whereas the third required no therapy, and was alive 102 months after diagnosis. All these three patients carried mutated IGHV genes. Interestingly, in a recent study by Best *et al.*[210] they have documented a subset of Binet stage A CLL patients with 17p-deletion and mutated IGHV genes having a stable disease for several years without requiring therapy. Our findings thus support the recent data by Best *et al.*[210]. Hence, *TP53* abnormalities, particularly in patients presenting with early-stage disease, do not necessarily result in an aggressive disease course and poor outcome in CLL.

To conclude, we confirmed the high prevalence of *TP53* mutations in 17p-deleted patients but observed a lower incidence of *TP53* mutations without 17p deletion in our population-based study compared to previous reports[206,208,209]. Our finding further supports the idea that *TP53* mutations are gained during the disease progression rather than at disease onset.

# CONCLUDING REMARKS

Somatic mutations of *TP53* that results in the absence or impaired function of p53 is one of the most important mechanism by which the p53 pathway is damaged during tumorigenesis. Moreover, loss of normal p53 function has been indicated to associate with unfavorable prognosis in both DLBCL and CLL. It has also been shown that polymorphisms in the *TP53* gene and genes involved in the p53 pathway might have potential clinical interests. The two most commonly described SNPs in the p53 pathway are the *TP53* codon 72 polymorphisms and *MDM2* SNP309, both of which have been reported to influence the function of p53 and Mdm2, respectively. In addition, aberrant DNA methylation, which result in transcriptional silencing of tumor-related genes, have been observed as the most consistent epigenetic changes in human cancers, including B-cell lymphomas. This has been implicated in the findings that promoter hypermethylation of $p16^{INK4a}$ potentially contributes to the pathogenesis and progression of DLBCL.

Overall, we confirmed the negative prognostic impact of *TP53* mutation in DLBCL, which was particularly prominent in the GC subtype (Paper I). However, no association was observed between *TP53* mutations and survival in non-GC DLBCL patients, suggesting that other markers, such as NF-κB, might play a role in protecting the cells from chemotherapy-induced apoptosis. Our data further highlights the importance to study the impact of *TP53* mutations in these two biologically distinct subgroups of DLBCL. Nevertheless, the present finding needs to be further investigated in R-CHOP treated materials, in order to test its reliability as a predictor of inferior clinical outcome, since the benefit of rituximab was found to be consistent in both GC and non-GC DLBCL.

On the other hand, we have shown that the *MDM2* SNP309 did not predict survival in either DLBCL or CLL (Papers I and III). Hence, we believe that the *MDM2* SNP309 should not be considered as a prognostic marker in these entities. Moreover, no prognostic impact of the *TP53* codon 72 polymorphism was observed in our series of DLBCL patients (Paper I). Thus, this polymorphism should also not be considered as a predictor of poor outcome in DLBCL.

We have, for the first time, analyzed the prognostic impact of $p16^{INK4a}$ methylation in DLBCL using the pyrosequencing technique (Paper II). In short, we could not confirm the correlation between the $p16^{INK4a}$ methylation and patients' survival, particularly with regard to LSS. Although we have documented a better PFS in young patients with >25% $p16^{INK4a}$ methylation level, and demonstrated this further in multivariate analysis, the significance of this, if any, needs to be further validated. Thus, we question the role of $p16^{INK4a}$ promoter methylation as a negative prognostic factor in DLBCL.

In Paper IV, we confirmed the negative correlation between the presence of *TP53* mutation with 17p-deletion and survival in newly-diagnosed CLL patients. However, the prognostic impact of *TP53* mutation without accompanying 17p-deletion could not be demonstrated due to the low number of *TP53*-mutated cases in our material. Nonetheless, our findings further emphasize that *TP53* mutations are rarely observed in early-stage CLL.

Altogether, the studies in this thesis have investigated the role of *TP53* mutation, *TP53* codon 72 polymorphism and *MDM2* SNP309, and $p16^{INKa}$ methylation in the survival of DLBCL and/or CLL. In conclusion, we could confirm the negative prognostic impact of *TP53* mutations in DLBCL, particularly in the GC subtype, whereas *MDM2* SNP309 and *TP53* codon 72 polymorphisms appear to lack clinical relevance. We also question the role of $p16^{INKa}$ methylation as a poor-prognostic factor in DLBCL. Finally, the presence of *TP53* mutation in CLL appears to be infrequent at disease onset and instead may arise during disease progression.

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