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**Molecular genetic studies on
Chronic Lymphocytic Leukemia and Acute Myeloid Leukemia
- with focus on prognostic markers**

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Abstract

The present thesis is focused on the prognostic value of genetic variations and alterations in the initiation and development of chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML) patients. Several prognostic markers based on genetic or chromosomal aberrations are today used in clinic in these heterogeneous diseases. Novel biomarkers have been identified through next generation sequencing techniques and some of them may be useful as prognostic markers in clinical diagnostic. In *papers I-IV* we have investigated some of this markers in CLL and AML tumor cells.

In *papers I and III* we investigated the prognostic value of the MDM2 SNP309 in relation to the presence of *TP53* mutations in tumor cells from CLL and AML patients. The SNP309 G-allele was associated with a shorter overall survival in *TP53* wildtype CLL and non-normal karyotype AML patients. Mutations in the *TP53* gene were found in 6.2% in CLL and 21.7% in AML and were always associated with adverse overall survival. This was most significant observed among the AML patients, where the three year survival was zero.

In *paper II* we investigated mutations in *NOTCH1* and *NOTCH2* as prognostic biomarkers in CLL. Notch1 and Notch2 play critical roles in lineage differentiation of white blood cells. We found mutation only in *NOTCH1* in a frequency of 6.7% and our analysis revealed a shorter overall survival for these. *NOTCH1* mutations were almost mutually exclusive with *TP53* mutations and represented together 12.9% in CLL patients, and they may both be strong prognostic biomarkers in CLL.

In *paper IV* we studied mutations in the tricarboxylic acid cycle. Metabolic disturbances in cancer cells have been known for many years, but recently mechanistic explanations have been identified. Hot spot mutations in *IDH1/2* genes, result in neomorphic enzyme activities that results in global hypermethylation of the cancer cell genome. We found mutations in 21% of the AML patients. Among the CN-AML patients there is a lack of prognostic markers and in this subgroup we found patients with *IDH2* mutations to have a shorter overall survival (3 vs. 21 months ($p=0.009$) for mutated and wild-type patients, respectively). Additionally, we also studied a SNP in the *IDH1* gene, and both the *IDH2* mutations and the SNP showed to have a potential as a new prognostic markers in CN-AML.

In summary, the results in *papers I-IV* have a potential to function as novel prognostic biomarkers in the clinic for therapeutic considerations and may also be targets for novel drugs for CLL and AML patients.

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List of papers

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- I** **Willander K**, Ungerback J, Karlsson K, Fredrikson M, Söderkvist P, Linderholm M. MDM2 SNP309 promoter polymorphism, an independent prognostic factor in chronic lymphocytic leukemia. Eur J Haematol. 2010 Sep;85(3):251-6.

- II** **Willander K**, Dutta RK, Ungerback J, Gunnarsson R, Juliusson G, Fredrikson M, Linderholm M, Söderkvist P. NOTCH1 mutations influence survival in chronic lymphocytic leukemia patients. BMC Cancer. 2013 Jun 4;13:274.

- III** **Jakobsen Falk I***, **Willander K***, Chaireti R, Lund J, Hermanson M, Gréen H, Lotfi K, Söderkvist P. TP53 mutations identify a subgroup of AML patients with dramatically impaired outcome. Submitted.

- IV** **Willander K**, Jakobsen Falk I, Chaireti R, Paul E, Hermanson M, Gréen H, Lotfi K, Söderkvist P. Mutations in the isocitrate dehydrogenase 1/2 genes and IDH1 SNP 105C>T have a prognostic value in acute myeloid leukemia. Manuscript.

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Abbreviations

2-HG	2-hydroxyglutarate
5-MeC	5-methylcytosine
ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
ATM	Ataxia telangiectasia mutated
BCR	B-cell receptor
bp	base pair
CDR	Complementary diversity region
CEBPA	CCAAT enhancer binding protein alpha
CIMP	CpG island methylator phenotype
CLL	Chronic lymphocytic leukemia
CLLU1	CLL up-regulated gene 1
CML	Chronic myeloid leukemia
CN-AML	Cytogenetically normal AML
CR	Complete remission
ddNTP	Didioxynucleotide
DNA	Deoxyribonucleic acid
dNTP	Dioxynucleotide
ELP	Early lymphocyte precursor
FISH	Fluorescence in situ hybridization
FLT3	Fms like tyrosine kinase 3
HSC	Hematopoietic stem cell
IDH	Isocitrate dehydrogenase
IGHV	Immunoglobulin heavy variable chain
ITD	Internal tandem duplication
MDM2	Murine double minute2
MDS	Myelodysplastic syndrome

MZB	Marginal zone B cell
NECD	Notch extracellular domain
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activate B cells
NICD	Notch intracellular domain
NPM1	Nucleophosmin1
OS	Overall survival
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SHM	Somatic hypermutation
SNP	Single nucleotide polymorphism
SSCA/SSCP	Single strand conformation analysis/polymorphism
TAD	Transactivation domain
T-ALL	T-cell acute lymphoblastic leukemia
t-AML	Therapy related AML
TET	Ten eleven translocation
TP53	Tumor protein p53 gene
ZAP70	Zeta-chain-associated protein kinase 70
α -KG	α -ketoglutarate

Introduction

Worldwide, leukemia accounts for 2.5% of all cancers with about 250000 people diagnosed each year (Rodriguez-Abreu et al., 2007). In Sweden, approximately 1000 individuals are annually diagnosed with leukemia, affecting both men and women in all ages (The Swedish Cancer Society) however, most of the cases occur in the elderly population. The incidence rate are slightly higher among men than women across all leukemia.

Leukemia is a cancer of the white blood forming cells (leukocytes) in the bone marrow and can be divided into acute and chronic leukemias. Depending of the cell type of origin, leukemia is divided into lymphoid or myeloid leukemia and can additionally be divided into four main types, acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL) (Rodriguez-Abreu et al., 2007). Acute leukemia is characterized of immature blast cells with blocked differentiation, while chronic leukemia is characterized by mature cells, which displace normal cells, leading to accumulation of abnormal cells in the bone marrow and the peripheral blood. The myeloid lineage differentiates into granulocytes, monocytes, thrombocytes and erythrocytes and the lymphoid lineage differentiates into B- and T- lymphocytes. In contrast to CLL and AML, the genetic alteration, which causes CML is identified to a translocation between chromosomes 9 and 22, the Philadelphia chromosome, and a fusion protein is formed (BCR/ABL) with constitutive tyrosine kinase activity. ALL is characterized of clonal excess of lymphoblasts in the bone marrow and mainly affects children.

Similar to other cancers, leukemia arise from normal cells in a multistep development. This results in alterations of the genome as mutations, insertions, deletions, translocations, amplifications or epigenetic changes. The alterations occur in genes, which control the function of the cell proliferation and differentiation (oncogenes) or in genes, which suppress growth and proliferation (tumor suppressor genes) or in genes, which control the DNA repair and apoptosis. Additional hallmarks, which are proposed in cancer development, are the possibility to evade the immune system, tumor promoting inflammation and changes in cellular metabolism (Hanahan & Weinberg, 2011).

The focus in this thesis has been to identify genetic alterations and markers for prognostication. These markers may clinically be used as prognostic markers for diagnosis, choice of and/or monitoring treatment. There is a great heterogeneity among CLL patients as well as AML patients and due to that it has been essentially to find novel prognostic markers. Genome-wide analysis through next generation sequencing techniques in tumor cells from both CLL and AML patients have identified novel biomarkers. In this thesis we have investigated some of this novel biomarkers in cohorts of CLL and AML patients.

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common leukemia among adults in the western world, but rare in Asia (Dighiero & Hamblin, 2008). In Sweden, approximately 500 new cases are diagnosed each year. The median age at diagnosis is about 70 years and it is rare in people younger than 50 years. The disease is nearly twice as common among men as women. The cause of CLL still remains unknown and most of the cases are sporadic. Familial CLL has been reported among first degree relatives, however no single inherited mutation has been identified. (Goldin et al., 2010). Agricultural workers exposed to herbicides have higher incidence of CLL (Dighiero & Hamblin, 2008).

CLL is a heterogeneous disease both from a biological and clinical point of view and so far incurable. Some patients survive for many years, more than 20 years, and require no or little treatment whereas others have an aggressive and rapidly progressive disease and need immediate therapy (Chiorazzi et al., 2005, Zenz et al., 2010). The disease is diagnosed as CLL when the peripheral blood consists of more than 5×10^9 B lymphocytes during a period for at least 3 months. The malignant cells are monoclonal, small, mature CD5+ B lymphocytes co-expressed with the B cell surface antigens CD19, CD20 and CD23 and low levels surface immunoglobulin, confirmed by flow cytometry (Hallek et al., 2008). The malignant cells appear in peripheral blood, bone marrow, lymph nodes and spleen. The disease is characterized by an accumulation of malignant non dividing cells most of them arrested in G₀ or early G₁ phase of the cell cycle. A small fraction are proliferating cells, where patients with greater proliferation rate have been found with a more aggressive disease (Zenz et al., 2010) and these patients also present symptoms including fever, fatigue, weight loss and night sweats (Inamdar & Bueso-Ramos, 2007).

Therapy

In general, asymptomatic early stage CLL patients should not be treated (“watchful waiting”), before the disease is in progression. No studies have shown that early treatment on asymptomatic CLL have long-term benefits compared to starting treatment when the disease present symptoms (Hallek et al., 2008). Since the mid 50’s the CLL patients have been treated with the alkylating agent chlorambucil, but few complete remissions were reached. Today, it is most elderly patients with comorbidity, who are treated with chlorambucil (Dighiero et al., 1998). Since the 90’s the first line treatment is fludarabine (F) (purine analog) in combination with cyclophosphamide (C) (alkylating agent) (Rai et al., 2000, Catovsky et al., 2007). Enhanced response measured as prolonged progression-free survival and overall survival is seen in previously untreated CLL patients by the adjuvant CD20 antibody rituximab to fludarabine (Byrd et al., 2005). CLL patients resistant to fludarabine treatment present an advanced disease, often with p53 abnormalities is treated with the monoclonal antibody alemtuzumab, a monoclonal antibody against CD52, which functions independently of an intact

p53 (Lozanski et al., 2004). Allogeneic stem cell transplantation (stem cells from a donor) should be considered in case of resistance to purine analog based therapy in eligible patients (Hallek et al., 2008).

Prognostic markers in CLL

Clinical markers

Two major stratification staging systems are used at diagnosis due to the tumor burden in CLL (Binet et al., 1981, Rai et al., 1975). The Binet system is divided into three stages (A, B and C) and the Rai system is divided into five stages (0-IV). Binet stage A and Rai stage 0 classified patients are associated with better prognosis and long overall survival (survival 9 years). The patients in Binet stage B and Rai stage I/II are in an intermediate risk group (survival 5years), whereas poor risk patients belong to Binet C and Rai III/IV with shorter overall survival (survival 2 years) (Hamblin, 2007). However, the disease is not stable and may change during the progression, thus other markers are needed to predict the clinical course and patients treatment response. In this thesis the patients are divided at diagnosis according to the Binet system (**Table 1**).

Table 1. Binet Staging

Stage	Number of involved lymph node areas	Hemoglobin g/L	Platelets x10 ⁹ /L	
A	<3	>100		>100
B	>3	>100		>100
C	any	<100	or	<100

Biological markers

IGHV mutational status

The basic structure of the immunoglobulin or antibody consists of two identical heavy chains and two identical light chains. The B-cell receptor (BCR) consists of the membrane bound immunoglobulin and the heterodimer CD79A/CD79B, essential for the signal transduction (Stevenson & Caligaris-Cappio, 2004). In response to an antigen, the B cell receptor in both normal and malignant cells, is designed through multiple variable combinations of the variable (V_H), diversity (D) and joining (J_H) gene segments in the immunoglobulin heavy chain and the immunoglobulin light chain variable (V_L) and joining (J_L) gene segments. The light (kappa or

lambda) chain lacks the D segment. There is about 50 functional V_H genes, 23 functional D gene segments and six J_H gene segments. Rearrangement of the V, D, and J genes is a recombination that first joins one of the D gene segments with one of J segments and then joining the combined DJ sequence with one of the V gene segments (**Figure 1**) (Jung et al., 2006). The light chains are constructed from two gene segments (V and J). By somatic hypermutation (SHM) of three complementary diversity regions (CDRs) the BCR selection is increased. The CDR3 region has the highest variability. This process occurs in germinal center (GC) in the secondary lymphoid organ, and SHM are introduced in a high rate in the immunoglobulin variable region and the cells undergo massive clonal expansion. The antigen selected cells differentiate into plasma cells or memory cells (Klein & Dalla-Favera, 2008, Zenz et al., 2010), thus each B cell is equipped with a unique B cell receptor which can be used as a molecular marker in B cells malignancies (Küppers et al., 1999).

Figure 1. Rearrangement of the immunoglobulin heavy chain variable gene.

Two groups, Hamblin et al., and Damle et al., demonstrated in 1999 independently of each other that CLL could be divided into two subsets depending on presence or absence of mutations in the immunoglobulin heavy variable chain gene (IGHV). Patients with mutated IGHV genes had a significantly longer survival compared with patients with unmutated IGHV genes that presented a more aggressive clinical course. They reported a median survival of about 9 years for patients with unmutated IGHV genes whereas median survival of about 24 years was reported for patients with mutated IGHV genes. Patients with mutated IGHV gene are also more often associated with stable disease, requiring no or little treatment as patients with unmutated IGHV gene are frequently associated with progressive disease (Hamblin et al., 1999, Damle et al., 1999). This has later been confirmed by several studies. A cut off of more than or equal to 98% similarity to the corresponding germline IGHV gene is classified as unmutated. The selection of a cut off at 98% is due to the commonly occurring polymorphisms in the IGHV genes (Tobin et al., 2005, Davi et al., 2008). IGHV gene mutational status is now

established and commonly used prognostic marker in CLL, as it is a reliable marker and does not change over time.

Studies of VH gene usage have shown a biased VH gene repertoire in CLL cells compared to normal B cells, such as IGHV1-69, IGHV3-7, IGHV3-21, and IGHV4-34 are more common in CLL cells than in normal B cells (Hamblin et al., 1999, Tobin et al., 2004). A study by Tobin et al. 2002 reported that IGHV3-21 usage in CLL represents a subset of cells associated with worse outcome, regardless of the mutational status. Moreover, presence of stereotyped CDR3 sequences indicating a role of antigen selection in the CLL development (Murray et al., 2008, Lanemo Myhrinder et al., 2008, Rosén et al., 2010).

The normal counterpart of the CLL cell is still unknown. Possibly the unmutated IGHV clone originate from cells which have not entered the germinal center and the mutated IGHV clone undergoes the malignant transformation after entering the germinal center indicating that the mutated cell of origin is more differentiated (Rodriguez-Vicente et al., 2013).

CD38 and ZAP70

To determine the IGHV mutational status is time consuming, so therefore surrogate biomarkers have been investigated. High expression of CD38, a transmembrane glycoprotein and cell surface marker, expressed during B cell development, correlates with unmutated IGHV gene. However, this biomarker is not stable during the course of the disease (Hamblin et al., 2002). A more reliable marker is the tyrosine kinase Zeta-chain-associated protein kinase 70kDa (ZAP70) (Crespo et al., 2003), where high ZAP70 levels predict for adverse outcome (Rassenti et al., 2004). However, this marker is not yet standardized because of the intracellular localization and the detection of ZAP70 with flow cytometry is difficult. Both CD38 and ZAP70 are useful markers independent IGHV mutation status (Hamblin, 2011). While IGHV mutational status is stable over time, cytogenetic changes and mutations affecting other genes frequently occur during the course of the disease.

Genetic aberrations

No common single genetic aberration has been found to be responsible for the disease development in CLL. With fluorescence in situ hybridization (FISH) the most common recurrent genetic aberrations in CLL can be detected in more than 80% of the CLL patients. Some patients have more than one cytogenetic defects, displaying the heterogenic nature of the disease. Five major prognostic categories have been defined (Döhner et al., 2000). The most common chromosomal aberrations are deletions in 13q14 in 40-60% of the CLL cases, 11q22-23 deletion in 15-20% and 17p13del in 5-10%. Trisomy 12q is detected in about 7-30% and about 20% of CLL patients display a normal karyotype. The frequency may vary between different studies. The study by Döhner et al., observed that the median overall survival for 17pdel patients were 32 months, for 11qdel were 79 months, trisomy 12q 114 months, and 111 and 133 months for normal karyotype and 13qdel, respectively (Döhner et al., 2000, Van Bockstaele et al., 2009).

The 17pdel is associated with a very aggressive clinical course. The tumor suppressor *TP53* gene is located at chromosome 17p and deletion of one allele is often followed by disruption of the remaining allele through mutation according to a prototypic two-hit hypothesis (Knudson, 1978). The p53 tumor suppressor protein plays a key role in inducing apoptosis and cell cycle arrest after DNA damage. Treatment with purine nucleoside analogues e.g. fludarabine or alkylating agents e.g. chlorambucil is based on the concept to upregulate an intact wildtype p53 protein stimulating apoptosis. CLL patients with 17pdel and/or *TP53* mutation are associated with treatment resistance and short survival (Döhner et al., 1995, Wattel et al., 1994, Rossi et al., 2009, Willander et al., 2010). Relapsed and refractory CLL show high frequencies of 17pdel/*TP53* mutation (Zenz et al., 2011).

The 11qdel is associated with poor survival and extensive lymphadenopathy especially in younger CLL patients (Döhner et al., 1997). The ATM (ataxia telangiectasia mutated) gene is located at chromosome 11q22 and the protein acts upstream the p53 protein as a response to DNA damage, stabilizing the p53 protein. Both deletions and/or mutations are present in the ATM gene (Austen et al., 2005, Bullrich et al., 1999) however, 30% of the patients with 11qdel do not have biallelic inactivation through ATM mutation, suggesting that BIRC3, a negative regulator of NF- κ B, also located at 11q22.2, may represent another candidate gene recently found to be mutated in CLL and associated with poor treatment response and overall survival (Rossi et al., 2012b).

Trisomy12 is found associated with early progression in CLL. Recurrent duplication in the area of 12q13 is found and there is an ongoing speculation of an oncogene playing a pathogenic role (Stilgenbauer et al., 2002). The MDM2 (murine double minute2) oncogene is reported to be overexpressed in CLL (Watanabe et al., 1994) and is located in the same region as the amplification unit of chromosome 12. However, MDM2 overexpression is not associated with the trisomy 12 aberration. CLL up-regulated gene (*CLLU1*) is highly up-regulated in CLL cells and also located in the same chromosome 12 region, but as for MDM2, the expression do not correlate with trisomy12 (Buhl et al., 2006). So far, the role of trisomy12 and which genes that may be involved is unsolved. Additionally, it is recently reported that *NOTCH1* (chromosome 9q34) mutations in CLL are associated with trisomy12 and together confer an unfavorable prognosis (Balatti et al., 2012, Del Giudice et al., 2012).

The 13qdel is the most frequent chromosome alteration in CLL and as the sole aberration it is associated with favorable prognosis (Döhner et al., 2000). Genes, which are deleted in 13q also remains to be determine. However, two micro RNA's, miR-15a and miR-16-1, are suggested as candidate genes, since they are deleted or down-regulated in the majority of CLL cases (Calin et al., 2002). Mir15/16 negatively regulate the anti-apoptotic protein Bcl-2 and promotes apoptosis, deleted or down-regulated mir15/16 correlates to overexpression of Bcl-2 often found in CLL (Cimmino et al., 2005).

Novel biomarkers

The genetic lesions detected by FISH do not fully clarify the heterogeneous clinical course, severe complications and chemoresistance. Frequent alterations recently discovered by whole

exome sequencing associated with leukemic tumorigenesis are found in the *NOTCH1*, *SF3B1* and *BIRC3* genes in approximately 5-10% of each gene in CLL patients at diagnosis (Fabbri et al., 2011, Puente et al., 2011, Rossi et al., 2011, Rossi et al., 2012b). Deletions in the *NOTCH1* gene cause impaired degradation and thus pathway activation. *FBXW7* gene is part of the SCF ubiquitin protein complex, targets the PEST domain of the Notch receptors for proteosomal degradation. Mutations in the *FBXW7* gene occur in a low frequency (Fabbri et al., 2011, Puente et al., 2011, Wang et al., 2011). The precise role of *SF3B1* mutations is unknown, but since SF3B1 is a component of the spliceosome and involved in splicing of genes controlling cell-cycle progression and apoptosis, which may explain its contribution to cell proliferation and survival (Rossi et al., 2011). BIRC3 is a negative regulator of NF-κB, and thus disruption of BIRC3 through mutation or deletion may constitutively activate the NF-κB pathway and up-regulate NF-κB target genes e.g. numerous antiapoptotic genes in CLL cells (Rossi et al., 2012b). Alterations in the *NOTCH1*, *SF3B1* and *BIRC3* genes are associated with poor survival and resistance to fludarabine treatment in patients harboring wildtype *TP53*. Additionally, low frequency of mutations in the *MYDD88* gene, an intracellular transducer of cytokine signaling and toll-like receptor activation, are also identified in CLL and described to alter the NF-κB inflammatory pathway. The clinical impact in CLL of mutations in the *MYDD88* gene are still unknown (Wang et al., 2011).

A study by Rossi et al. 2013 suggested an integrated model of cytogenetic (Döhner et al., 2000) and mutation analysis to categorize newly diagnosed CLL patients in four subgroups according to risk of death. The high-risk group included patients with 17pdel/*TP53* mutations and/or BIRC3del/*BIRC3* mutations, in the intermediate group the patients harboring *NOTCH1* mutation and/or *SF3B1* mutation and/or 11qdel were included. The low-risk group patients included those with trisomy12 and with normal cytogenetic and no mutations at all and the last subgroup is the very low-risk category, which included patients harboring del13q as the sole genetic aberration. The results of Rossi et al. (2013) are summarized in **Table 2**.

Table 2. Integrated mutational and cytogenetic alterations in CLL

Risk Group	Aberration	5 year OS %	10 year OS %
High	TP53 mut/del, BIRC3 mut/del	50.9	29.1
Intermediate	NOTCH1 mut, SF3B1 mut, del11q	65.9	37.1
Low	Trisomy12, None	77.6	57.3
Very low	del13q	88.9	69.3

Acute Myeloid Leukemia

In Sweden, approximately 350 people in all ages are diagnosed in acute myeloid leukemia every year. AML is more common in the elderly with a median age at diagnosis about 70 years. The

underlying mechanisms for AML are only partly known, and several environmental and genetic risk factors have been associated with development of AML, as occupational exposure to organic solvents such as benzene and cigarette smoking. AML sometimes appears secondary to chemotherapy for other types of tumors (t-AML) (Rodriguez-Adreu et al., 2007). AML may also arise from myelodysplastic syndrome (MDS) or myeloproliferative disease so called secondary AML. Genetic disorders such as Down syndrome, Fanconi anemia, or Bloom syndrome predispose for AML via genomic instability (Popp & Bohlander., 2010). Additionally, the age of the patients, represents another independent risk factor, older patients commonly have adverse prognosis, as they have higher frequency of high-risk cytogenetics (Grimvade et al., 2001, Juliusson et al., 2009).

AML is characterized by an accumulation of genetic alterations in hematopoietic progenitor cells affecting self-renewal, proliferation and differentiation resulting in accumulation of blast cells in peripheral blood and bone marrow (Döhner & Döhner, 2008). AML has a rapidly progression and the disease is fatal within weeks or months if left untreated. The 5-year overall survival is 35-40% and less than 15% of AML patients above 60 survive (Stone, 2002). Some characteristic symptoms are fatigue and shortness of breath due to anemia, bleeding and easy bruising due to thrombocytopenia, and infections due to leukopenia.

Therapy

The majority of AML patients receive intensive induction chemotherapy as soon as possible after diagnosis, including a combination of anthracycline (daunorubicin or idarubicin) and cytarabine to achieve complete remission (CR). The standard induction therapy is three days of an anthracycline and seven days of cytarabine (“3+7”). Of younger adults, 60 to 80% reach CR, still the majority of the patients are not cured and relapse. Patients belonging to the favorable risk group are generally treated with conventional therapy, while patients in the risk group with adverse prognosis should undergo stem cell transplantation if they are eligible (**Table 3**) (Döhner et al., 2010, Martelli et al., 2013).

Prognostic markers in AML

Cytogenetic and molecular risk markers

The most important prognostic parameters in AML is so far to stratify the disease based on proliferation rate, complete remission, and risk of relapse, overall survival and sensitivity to chemotherapy into three major cytogenetic and molecular risk groups according to European LeukemiaNet (ELN) (Döhner et al., 2010): favorable, intermediate and adverse prognosis (**Table 3**). Translocations, inversions and deletions are common in AML. Mutations in *NPM1*,

CEBPA and *FLT3* genes have been incorporated as prognostic markers into the classification of risk groups (Döhner et al., 2010). The favorable group: t(8;21)(q22;q22) and inv(16)(p13q22) or t(16;16)(p13;q22) resulting in fusions of *RUNX1/RUNX1T1* and *CBFB/MYH11* genes respectively, which results in impaired myeloid differentiation. Mutated NPM1 and double mutated CEBPA in absence of mutated FLT3-ITD also belong to the favorable group. The adverse risk group: inv(3)(q21q26)/t(3;3)(q21;q26), del(5q)/-5, del(7q)/-7, del(17p), and complex karyotypes, defined as at least three chromosome aberrations. Complex karyotype occurs in 10-12% of the patients and is associated with very poor outcome. FLT3-ITD with normal karyotype and in absence of NPM1 mutation is a molecular marker with worse prognosis and is therefore grouped in the adverse risk category (Grossmann et al., 2012). The intermediate risk group is the largest and constitute about 45% of de novo AML patients with cytogenetically normal karyotype (CN-AML), however this group is heterogeneous where some patients reach complete remission and become long time survivors, while others rapidly relapse with aggressive or resistant disease. Thus, treatment decision has been difficult, and further stratification of the heterogeneous intermediate risk group is warranted for choice of adequate and efficient therapy. The Swedish AML guidelines are almost the same as ELN.

Table 3. Cytogenetic and molecular risk groups according to European LeukemiaNet	
Genetic group	Subsets
Favorable	t(8;21)(q22;q22); <i>RUNX1/RUNX1T1</i> inv(16)(p13q22) or t(16;16)(p13;q22); <i>CBFB/MYH11</i> Mutated NPM1 without FLT3-ITD (normal karyotype) Mutated CEBPA (normal karyotype)
Intermediate	Mutated NPM1 and FLT3-ITD (normal karyotype) normal karyotype, mutated NPM1 t(9;11)(p21;q23); <i>MLL/MLLT3</i> Cytogenetic abnormalities not classified as favorable or adverse
Adverse	Wild-type NPM1 and FLT3-ITD (normal karyotype) inv(3)(q21q26)/t(3;3)(q21;q26); <i>RPN1-EVI1</i> t(6;9)(p23;q34); <i>DEK-NUP214</i> t(v;11)(v;q23); <i>MLL</i> rearranged del(5q)/-5, del(7q)/-7, abnl(17p); complex karyotype

The FLT3 tyrosine kinase receptor plays an important role in proliferation, survival and differentiation of hematopoietic cells. Internal tandem duplications (ITD) of the *FLT3* gene gives a constitutive activation of tyrosine kinase phosphorylation. The duplication of the *FLT3* gene is common in AML, approximately 30%, and is associated with adverse prognosis (Döhner, 2007). A point mutation in the tyrosine kinase domain is present in about 7% in the *FLT3* gene, also given a constitutive activation of tyrosine kinase activity (Mead et al., 2007).

Nucleophosmin1 (NPM1) shuttles between nucleus and cytoplasm and is involved in several cellular processes, as stability and transcriptional activity of p53 after different types of stress,

as NPM1 functions as a positive regulator of ARF protein stability. Mutations in the *NPM1* gene resulting in abnormal accumulation of the NPM1 protein in the cytoplasm and functional impairment of the ARF tumor suppressor pathways by preventing ARF to bind to MDM2 at cellular stress. Mutations are found in 45%-62% in CN-AML and is associated with favorable prognosis as the sole mutation. Mutated *NPM1* simultaneously with mutated *FLT3* is associated with intermediate risk in CN-AML (Döhner et al., 2005, Grisendi et al., 2006).

CCAAT/enhancer-binding protein alpha (CEBPA) gene encodes a transcription factor critical for neutrophil development. Mutations in the *CEBPA* gene are found in about 10-15% in AML CN-AML and in 85% of the cases *CEBPA* has double mutations, which seemed to be consistent with better outcome (Dufour et al., 2010).

KIT is a tyrosine kinase receptor and play a key role in proliferation of normal hematopoietic progenitor cells. Mutations in the KIT gene in found in about 30% and associated with inferior outcome in several studies (Döhner & Döhner, 2008).

Other molecular mutations found in AML, but the clinical impact is unclear; Wilms tumor 1 (WT1), mutated in about 10%, is a transcription factor involved in regulation of apoptosis, proliferation and differentiation of hematopoietic progenitor cells (Döhner & Döhner, 2008). RUNX1, a transcription factor involved in normal hematopoietic differentiation and mutations are found in about 10% in CN-AML (Döhner & Döhner, 2008).

Novel biomarkers in AML

In recent years new techniques, as whole genome sequencing, exome sequencing and SNP array analysis, have been used to identify recurrent mutations, which can further stratify the disease (Mardis et al., 2009, Ley et al., 2010, Delhommeau et al., 2009, Döhner & Gaidzik, 2011). A number of these new recurrent mutations disturb the normal function of epigenetic regulation of transcription, such as mutations in the *IDH1*, *IDH2*, *TET2* and *DNMT3A* genes, however these mutations are not yet validated as prognostic markers. *DNMT3A* (DNA nucleotide methyltransferase 3A) encode for methyltransferase, an enzyme that catalyze addition of a methyl group to cytosine at CpG dinucleotide. DNA methylation is an important regulator of gene expression, thus abnormal methylation is consistent with tumorigenesis. In AML, the frequency of *DNMT3A* mutations is about 20%, however it is not known in what way the mutations contribute to the disease (Ley et al., 2010). *TET2* encodes for a protein involved in converting 5-methylcytosine to 5-hydroxymethylcytosine, an important step in DNA demethylation. Mutations in *TET2*, block the DNA demethylation. *TET2* mutations occur in about 7-23% in AML (Delhommeau et al., 2009, Abdel-Wahab et al., 2013). Similar to *TET2* mutations, *IDH1/2* mutations interfere with TET2 mediated demethylation and a global DNA-hypermethylation, often referred as CpG Island Methylation Phenotype (CIMP).

Genes investigated in the present thesis

The p53 tumor suppressor and the MDM2 oncogene

The p53 protein was first described in 1979 (Lane & Crawford 1979, Linzer & Levine, 1979) and in an interaction between large T-antigen of the simian virus (SV40), it was believed to be an oncogene, since high levels of p53 was found in tumor cell lines co-expressed with the Ras oncogene. Later studies showed that it was the mutated form of p53, which first had been studied. In 1989, p53 was recognized as a tumor suppressor and immense studies showed p53 to be among the most important tumor suppressor proteins of the cell, sometimes called “Guardian of the genome” (Lane, 1992).

The p53 protein consists of 393 amino acids (aa) and the protein migrates as a band at 53kD in gel electrophoresis, which has given its name. The p53 protein is encoded by the *TP53* gene, which consists of 11 exons, located on chromosome 17p13.1 (reviewed in Levine & Oren, 2009). The p53 protein can be divided in some well-defined regions, the amino terminal part (aa 1-97), which includes the transactivation domain with the binding site for MDM2 (murine double minute2) and a proline rich domain, required to activate apoptosis (Sakamuro et al., 1997). The central DNA binding domain (aa 98-292) is responsible for consensus sequence-specific DNA binding to transcriptional target gene promoters (exon 5-8 are located in the central core). Finally, the carboxy terminal domain (aa 300-393), includes the tetramerization domain (aa 300-355) and the C-terminal regulatory domain (aa 363-393) domain, regulating the core DNA-binding domain (**Figure 2**) (Vousden & Lu, 2002, Nag et al., 2013).

Figure 2. A schematic structure of the p53 protein.

The MDM2 oncogene, corresponding to the human HDM2, was first identified in a transformed mouse cell line 3T3-DM with amplified small DNA fragments (double minutes) and in 1992 MDM2 was shown to negatively regulate p53 (Momand et al., 1992). The MDM2 gene is mapped to chromosome 12q13-14 and the protein consists of 491 amino acids and contains two promoter elements, P1 and P2. In unstressed cells, MDM2 constantly monoubiquitinates p53 and the basal transcription of MDM2 is induced via the first promoter. In stressed cells, p53 induces and regulates transcription of many target genes, including the MDM2 gene. The p53 protein then binds to the second promoter of the MDM2 gene and up-regulates MDM2 transcription (Hu et al., 2007). MDM2 is a master regulator of the p53 protein to reduce the

activity of p53 by marking p53 by ubiquitin at several lysine residues for proteolysis after a stress response. (Momand et al., 2000).

The p53 signaling pathway

Tumor-suppressor genes are essential to keep cells under control, as acting by inhibiting cells progress through the cell cycle and thereby preventing cell proliferation, or by promoting apoptosis. When tumor-suppressors fail to function uncontrolled cell growth arise (Vogelstein et al., 2000). As a response to cellular stress, such as DNA-damage or exposed to chemotherapeutic agents, ubiquitin mediated degradation of p53 ceases and p53 accumulates in the cell and acts as a transcription factor, transactivating an array of genes involved in DNA repair, cell-cycle arrest and apoptosis. In unstressed cells, the p53 levels normally are very low and controlled by the negative regulator MDM2. In the absence of stress, p53 and MDM2 are linked to each other through an autoregulatory negative feedback loop that maintains low p53 levels. Thus, the p53 protein, plays a key role for protecting the organism from DNA damaging exposure and mutation and ultimately to tumor development. In response to DNA damage or other stress signals, p53 is phosphorylated by the ATM/ATR kinases resulting in phosphorylation of p53 on ser15, resulting in disruption of the MDM2-p53 interaction subsequently by abortion of degradation, stabilization and increased cellular p53 levels. The tumor suppressor p14ARF, a direct inhibitor of the E3 ligase activity of MDM2, is upregulated in response to oncogenic signaling in stressed cells, and blocks MDM2 to shuttle between nucleus and cytoplasm, preventing p53 ubiquitination and degradation (Pomerantz et al., 1998, Moll & Petrenko, 2003). Increased p53 levels regulated hundreds of genes, induce transcription of proteins promoting growth arrest (p21 also known as WAF1), apoptosis (Bax, PUMA and NOXA) or DNA repair (GADD 45). P53 can also act in an independent transactivation pathway via mitochondrion, block the anti-apoptotic protein Bcl2 and subsequently induce the release of cytochrome c and caspase activation of the apoptotic caspase cascade and apoptosis (**Figure 3**) (Bode & Dong, 2004).

Figure 3. The p53 signaling pathway. Upon stress signals, the p53 protein is phosphorylated by kinases, which inhibit the interaction of p53-MDM2, resulting in stabilization and activation of the 53 protein in the nucleus where it interacts with DNA sequence specific target genes leading to cellular response. (Adapted from Bode & Dong, 2004. Reprinted with permission from Nature Reviews Cancer).

Inactivation of the p53 pathway

The p53 protein is frequently inactivated by mutations in cancer cells. The importance of an intact *TP53* gene and p53 function is seen in Li-Fraumeni syndrome with early cancer onset due to *TP53* heterozygous germline mutations (Malkin, 2011). In solid tumors the *TP53* gene is mutated or deleted in about 50% of all tumors, but in leukemia, aberrations of the *TP53* gene is infrequent, ranging from 5-10% at diagnosis (Peller & Rotter, 2003). Mutations in the *TP53* gene occur mostly in the DNA binding domain (exon 5-8), as missense or nonsense mutations, deletions or inserts. The p53 protein functions as a tetramer and the mutant p53 and the wildtype p53 form a tetrameric complex and the mutant p53 act as a dominant negative inhibitor of the wildtype p53 (loss-of-function) (Vousden & Lu, 2002). Mutated p53 protein is unable to bind and transactivate target genes to promote apoptosis, DNA repair or cell cycle arrest. Instead, the mutated p53 protein gets new properties, as transactivation of other genes beneficial for the tumor, as the oncogene MYC, which induce increased proliferation (gain-of function) (Dittmer

et al., 1993, Bode & Dong, 2004). However, there may be other mechanisms to attenuate the p53 signaling, such as high levels of MDM2 through gene amplification/overexpression (Watanabe et al., 1994) or single nucleotide polymorphism (SNP) in the promoter region of the MDM2 gene (Bond et al., 2004, Gryshchenko et al., 2008, Willander et al., 2010). Further, mutations/deletions in the ATM gene is also able to decrease activation of p53. ATM is activated in response to DNA breaks and the consequence of ATM dysfunction leads to impaired p53 activation. Ataxia telangiectasia is a disorder with germline ATM mutations with predisposition for the development of lymphoid malignancies (Pettitt et al., 2001, Austen et al., 2007).

***TP53* mutations in CLL and AML**

In CLL, p53 may be inactivated through 17pdel in 5-7% at early stages and at later stages in 25-40% and in 40-50% in fludarabine refractory disease (Döhner et al., 2000). There is a strong correlation between 17pdel and *TP53* mutations, deletion in one allele is in most cases consistent with mutation of the remaining allele. However a fraction of CLL patients carry *TP53* mutations without 17pdel and vice versa (Rossi et al., 2009, Zenz et al., 2008). Studies have confirmed that about 4-12% have *TP53* mutations without accompanying 17pdel. Thus, this subgroup with *TP53* mutations but without 17pdel may belong to the same risk category as CLL patients with 17p deletion and provide prognostic information independent of 17pdel (Zenz et al., 2010, Rossi et al., 2013). Patients with *TP53* mutations are not recognized at diagnosis by routine diagnostic screening with FISH. Like patients with 17pdel, patients with *TP53* mutations predict for poor survival and resistance to chemotherapy (Rossi et al., 2009). Among patients with chemotherapy refractory disease, about 40-50% of the patients have lost one 17p allele or have a mutated *TP53* gene with reduced overall survival. More than 95% of the identified *TP53* mutations are found in exon 5-8, encoding the DNA binding domain (Rossi et al., 2009).

In de novo AML, loss of the short arm of chromosome 17 is reported in 5-10% (Stirewalt et al., 2001, Seifert et al., 2009), however the deletion is most accompanied with complex aberrant karyotypes and are rare in other subgroups. As in CLL, 17pdel in one allele is most often found associated with *TP53* point mutations in the remaining allele with adverse overall survival and resistance to conventional chemotherapy. Haferlach et al., 2008 and Rücker et al., 2012 have reported *TP53* mutations rate as high as 60-70%, respectively, in AML with complex karyotypes and most often associated with deletions of chromosome 5, 7 and 17p. *TP53* mutations in AML in any of the risk groups are suggested to represent a specific subset with most unfavorable outcome, displaying a very short OS (Grossmann et al., 2012).

Single nucleotide polymorphism

Naturally genetic variations of the human genome is estimated to more than 20 million positions (1000 Genome project) as single nucleotide polymorphisms (SNPs), and are found in both

coding and non-coding regions. SNPs, which are located in coding or regulatory areas of the genome may influence protein-levels and function, such as transcription factors, predisposition to diseases, response to drug treatment, and interaction with other mutations (Bond et al., 2005). The *TP53* gene contains several polymorphisms. One of the most frequent and studied SNP's is in exon four, codon 72 with an amino acid exchange from arginine to proline proposed to decrease p53 mediated apoptosis (Whibley et al., 2009, Sturm et al., 2005). Another well-characterized SNP in the p53 pathway is a regulatory SNP in the MDM2 gene (rs2279744) resulting in enhanced MDM2 levels and enhanced degradation of cellular p53 levels (Bond et al., 2004).

MDM2 SNP309

MDM2 is a key regulator of cellular p53 levels and is often amplified or overexpressed in a large number of human cancers that express wildtype p53. Decreased p53 activity by MDM2 amplification or *TP53* mutations influence tumor development. A SNP at position 309 in the first intron in the second promoter (P2) of the human MDM2 gene (MDM2 SNP309) with a T to G change has been associated with increased cancer risk with wildtype p53 in many different types of tumors in several studies (**Figure 4**) (Bond et al., 2004, Bond et al., 2007, Post et al., 2010a).

Figure 4. The SNP309 of the MDM2 gene. (Adapted from Arva et al., 2005).

In vitro studies have shown that the G allele binds with a higher affinity to the SP1 transcription factor than the T allele and is significantly associated with higher levels of MDM2 mRNA and protein, which increases the basal MDM2 levels and may attenuates the p53 function (Bond et al., 2004). A study in mice carrying the human MDM2^{SNP309T} or the MDM2^{SNP309G} allele and wildtype *TP53*, was performed to determine the impact of the SNP309 in tumorigenesis. Mice harboring two G alleles of the human MDM2 SNP309 gene were found to have increased MDM2 mRNA and protein levels. After ionizing radiation, the p53 response was reduced in the cells with MDM2^{SNP309G} alleles compared to the cells with MDM2^{SNPT/T}, and the mice harboring the G allele had decreased survival compared to the mice carrying the homozygous T allele (Post et al., 2010b). In addition, carriers of the Li Fraumeni syndrome germline p53 mutation and the SNP G-allele are associated with accelerated tumor formation displaying an additive effect of carrying both the SNP309 G-allele and *TP53* mutations (Ruijs et al., 2007).

Several studies have been reported about the MDM2 promoter polymorphism in solid tumors as well as in hematologic malignancies, however with conflicting results.

The structure and the function of the Notch receptor

Notch was first described in 1917 in *Drosophila melanogaster* with a notched phenotype at the ends of the wings, but the structure and function were unknown until the 1980s when the gene was cloned and identified as the Notch receptor (Wharton et al., 1985, Radtke & Raj, 2003). The Notch receptor has coined the downstream pathway and the mammalian Notch family consists of four members, Notch1-4, of transmembrane receptors that function as ligand-activated transcription factors. The receptors and the pathway regulate important functions in controlling cell fate, proliferation, differentiation and apoptosis (Radtke et al., 2004, Grabher et al., 2006). The human *NOTCH1* and *NOTCH2* genes share high structural similarities consisting of 34 exons. The receptor can be divided in Notch extracellular domain (NECD), transmembrane domain (TD) and Notch intracellular domain (NICD). The NECD of Notch1/2 consists of 36 epidermal growth factor (EGF) repeats. The number of EGFs repeats in Notch3/4 are 34 and 29 repetitive units, respectively. The EGF repeats facilitate ligand binding when Notch attaching on the surface of a neighboring cell. The EGFs repeats are followed by three cysteine-rich Notch/LIN12 repeats preventing ligand-independent signaling and linked to the heterodimerisation domain (HD). The NICD consists of a RAM domain (RBPjk Association module with the transcription factor complex CSL) followed by seven ankyrin-repeats (ANK-repeats), a transactivation domain (TAD) and a conserved proline/glutamic acid/serine/threonine-rich motif (PEST) harboring degradation signals (**Figure 5**) (Kopan et al., 2009, Grabher et al., 2006).

Figure 5. A schematic structure of the Notch1 receptor.

The signaling is mediated through the Notch receptor and a ligand attached to the surface of a neighboring cell. Five different Notch ligands have been identified in mammals, Delta1, 3-4, belonging to the Delta-like family and Jagged1 and 2 (belonging to the Jagged family) (Radtke et al., 2004).

Notch1 has an important role in regulating hematopoietic stem cell (HSC) maintenance through self-renewal and plays an essential role for normal T-cell development. Early lymphocyte precursors (ELP) differentiates into either B or T cells. The B cell development occurs in the bone marrow and when Notch1 signaling is absent and critical for B cell maturation, its

expression favors the T cell maturation in thymus. Notch2 is required for marginal zone B cells (MZB) differentiation (Radtke et al., 2004). Studies have shown that *NOTCH1* signaling is present in B cells promoting the terminal differentiation to antibody secreting cells in mature B lymphocytes (Santos et al., 2007).

The Notch signaling pathway

The extracellular and intracellular domains of the Notch receptor are generated from a pre-Notch protein and a series of modifications are required for the Notch receptor to be in an active form. Furin-like protease cleavage (S1 cleavage) and glycosylation of the EGF-repeats by glycosyltransferase Fringe family in the Golgi apparatus results in a heterodimeric receptor translocated to the cell membrane (Grabher et al., 2006). The glycosylation is important for the ligand specificity and following receptor-ligand binding, the Notch receptor first undergoes a S2 cleavage by ADAM (a disintegrin and metalloproteinase domain) proteinase in the extracellular domain, followed by a S3 cleavage by a γ -secretase complex in the transmembrane domain (Brou et al., 2000, Mumm et al., 2000). The liberated intracellular part of Notch, then translocates to the nucleus where it binds to the transcription factor CSL (CBF1/RBPj κ /Su(H)/Lag-1) and the Mastermind-like proteins (MAML) to dislocate co-repressors (CoR) and recruits co-activators (CoA). Additional co-activators as p300 forms a complex with Notch/CSL/Mastermind (Grabher et al., 2006), and initiates transcription of multiple targets genes such as MYC and NF- κ B (Palomero et al., 2006, Villimas et al., 2007). The C-terminal of the intracellular domain consists of a PEST region (a polypeptide rich in proline, glutamate, serine and threonine), which is important for ubiquitination by the E3-ligase FBXW7, a part of SCF ubiquitin ligase complex, and proteasomal degradation, limits the duration of the Notch activity (**Figure 6**) (Welcker & Clurman, 2008).

Figure 6. The Notch signaling pathway. **a:** Pre-Notch. **b:** Pre-Notch is cleaved into extracellular and intracellular domain. **c:** The Notch receptor binds to a ligand at an adjacent cell. **d:** ADAM proteinase cleavage in the extracellular domain and a second cleavage within the transmembrane domain by a γ -secretase complex. **e:** The liberated intracellular Notch translocates to the nucleus and interacts with a transcription complex and initiates transcription of multiple target genes. **g:** Ubiquitylation and proteasome degradation of intracellular Notch. (Adapted from Grabher et al., 2006. Reprinted with permission from Nature Reviews Cancer).

Notch mutations in hematologic malignancies

The oncogenic role of Notch was first identified in a minor fraction (<1%) of human T-cell acute lymphoblastic leukemia (T-ALL), since the Notch1 gene was involved in the chromosomal translocation in t(7:9)(q34;q34.3) (Reynolds et al., 1987). Later, activated *NOTCH1* mutations were found in both the heterodimerisation domain and the C-terminal PEST region in more than 50% in T-ALL (Weng et al., 2004). Mutations in the Notch heterodimerisation domain are thought to favor ligand independent signaling and increased intracellular Notch pathway activation (Koch & Radkte, 2007). Mutations in the Notch C-terminal PEST region enhanced protein half-life. Truncating stop codon eliminates PEST and no ubiquitination occur (Weng et al., 2004). Oncogenic Notch signaling is also suggested to play a role in other hematologic malignancies than T-ALL as Hodgkin's lymphoma, Multiple Myeloma (Leong & Karsan, 2006) as well as splenic marginal zone lymphoma (Rossi et al., 2012c, Kiel et al., 2012) and recently also in chronic lymphocytic leukemia (Puente et al., 2011, Fabbri et al., 2011, Willander et al., 2013). Thus, abnormal Notch signaling seems to be involved in development of B cell malignancies. Additionally, studies have shown constitutively activated signaling of both *NOTCH1* and *NOTCH2* in CLL cells but not in normal B lymphocytes. The same study also showed an increased NFκB activation and cell survival NOTCH signaling is activated (Rosati et al., 2009).

Notch1 mutations in CLL

Through next generation sequencing, the *NOTCH1* gene (located at chromosome 9q34) was detected with recurrent mutations in CLL by two independent groups (Fabbri et al., 2011, Puente et al., 2011). They found *NOTCH1* mutated in a frequency of 8.3% and 12%, respectively. The presence of *NOTCH1* mutations in CLL is later confirmed by several studies and frequencies ranging from 4.7% to about 12% depending on the study cohort. Mutations are reported to associate with a shorter overall survival and chemotherapy refractory disease (Rossi et al., 2012a, Shedden et al., 2012, Mansouri et al., 2013, Willander et al., 2013). *NOTCH1* mutations are more frequently associated with unmutated than mutated immunoglobulin genes (IGHV) and *NOTCH1* mutations are commonly linked with trisomy 12 (Baletti et al., 2012, Del Giudice et al., 2012). Significantly higher frequency of *NOTCH1* mutations are also reported in CLL clonally related Richter syndrome (RS), an aggressive lymphoma, developed from CLL. This may partly explain the poor prognosis associated with *NOTCH1* mutations in CLL, where 40-50% of *NOTCH1* mutated patients may develop clonally related RS (Puente et al., 2011, Rossi et al., 2012d). The *NOTCH1* mutations in CLL is almost always a 2 bp CT-deletion in exon 34 in the C-terminal PEST domain, generating a frameshift with subsequent stopcodon, which gives rise to a truncated protein eliminating the PEST sequence and this results in decreased proteasomal degradation and constitutive Notch activation of target genes, including MYC and NF-κB (Palomero et al., 2006, Villimas et al., 2007).

Isocitrate dehydrogenase 1 and 2 mutations in AML

Cellular metabolism is proved to play a central role in many cancers. This was proposed by Otto Warburg more than 80 years ago. He noted that proliferating cancer cells produced pyruvate and lactate, but reduced amount of ATP despite no restriction of oxygen (Weinberg & Chandel 2009). Cancer associated mutations in three metabolic enzymes are recently identified. Two of these enzymes, fumarate hydratase (FH) and succinate dehydrogenase (SDH), have been recognized in numerous of cancers, as renal cell carcinomas and paragangliomas (Losman & Kaelin, 2013). The third enzyme in the tricarboxylic acid (TCA) cycle suggested to associate with tumorigenesis, is isocitrate dehydrogenase (IDH). The first diseases to be identified with heterozygous mutations in the isocitrate dehydrogenase 1 (*IDH1*) gene were in glioma and AML tumor cells (Parsons et al., 2008, Mardis et al., 2009). Further studies have shown mutations in the homologous isocitrate dehydrogenase 2 (*IDH2*) gene (Marcucci et al., 2010) and a number of subsequent analyses have confirmed recurrent *IDH1/2* mutations in AML tumor cells (Boisel et al., 2010, Paschka et al., 2010, Wagner et al., 2010). The IDH family of proteins exists in three isoforms, where IDH1 is located in the cytosol and IDH2 and IDH3 are present in the mitochondrion. So far no mutations have been found in the *IDH3* gene (Dang et al., 2010).

In the TCA cycle, the wildtype IDH1 and IDH2 are in their normal functions involved in oxidation of isocitrate to the unstable intermediate oxalosuccinate and NADP^+ is reduced to NADPH. In the next step the β -carboxylgroup is released and carbon dioxide is formed and released during production of α -ketoglutarate (α -KG). Mutant IDH1/IDH2 gains a novel or enhanced enzymatic activity to catalyze α -ketoglutarate to 2-hydroxyglutarate (2-HG). Structurally, α -KG and 2-HG are very similar and the only difference is that a ketone group in α -KG is reduced to a hydroxyl group in 2-HG at the same position (**Figure 7**) (Losman & Kaelin, 2013, Cairns & Tak, 2013).

Figure 7. Wildtype IDH1/IDH2 catalyze isocitrate to α -KG and mutant IDH1/IDH2 convert α -KG to 2-HG. (Adapted from Cairns & Tak, 2013. Reprinted with permission).

The IDH1/2 mutants act as oncogenes and cause a gain-of-function alteration, promoting proliferation, and inhibiting differentiation and contribute to tumorigenesis. The 2-HG levels are very low in normal cells but increase dramatically with high levels in mutant IDH AML cells (Ward et al., 2010, Gross et al., 2010). The function of 2-HG in normal cells are unknown (Cairns & Mak, 2013). A study by Figuera et al., 2010 have shown that high levels of the 2-HG metabolite is associated with epigenetic alterations by inducing global DNA hypermethylation and impaired differentiation in hematopoietic cells. 2-HG is described as a competitive inhibitor

of α -KG (Xu et al., 2011). Ten-eleven translocation 2 (TET2) is a DNA demethylase enzyme and requires α -KG for hydroxylat 5-methylcytosine (5-MeC) as a step in the demethylation of DNA. Abnormal DNA methylation occurs in IDH1/2 mutant AML cells when production of high 2-HG levels compete with α -KG for TET2 and inhibit the demethylation (Figuera et al., 2010, Ito et al., 2010). A mutant TET2 phenotype generate the same aberrant DNA methylation reaction as mutant IDH1/2 phenotype (Cimmino et al., 2011). Furthermore, *IDH* mutations are mutually exclusive with *TET2* mutations and *IDH1* mutations are mutually exclusive with *IDH2* mutations in AML cells (Gaidzik et al., 2012).

Mutations in the *IDH1* gene occur in exon 4, codon 132 and in the *IDH2* gene in exon 4, codon 140 and 172 and all mutations generate a substitution of an arginine residue. *IDH1/2* mutations are found in a higher frequency in CN-AML than in cytogenetically abnormal AML patients. In unselective AML, *IDH1* mutations occur in a frequency of 5.5%-10.4% and *IDH2* R140 and R172 in an incidence of 6.1%-17.7% and 2.3% respectively. The clinical value of *IDH* mutations are not clear. While several studies show a negative impact in overall survival others suggest an improved or no effect on overall survival. In any case *IDH* mutations are most associated with CN-AML patients (Rakheja et al., 2012).

Aims

The general aim of the thesis was to investigate potential biomarkers with clinical prognosis in chronic lymphocytic leukemia and acute myeloid leukemia.

Paper I

To determine the prognostic role of the genetic variation of MDM2 SNP309 in CLL patients and correlate the genotype data with prognostic markers as *TP53* gene status, IGHV gene status and other clinical data.

Paper II

To determine the mutational activation of the *NOTCH1* and *NOTCH2* genes and elucidate the prognostic value in CLL patients and also correlate the *NOTCH* mutations to other biomarkers and clinical data.

Paper III

To determine the prognostic role of the genetic variation of MDM2 SNP309 and genetic alterations in the *TP53* gene and evaluate the clinical significance in the different risk groups and in relation to karyotype data in AML patients.

Paper IV

To determine the connection of mutations in the isocitrate dehydrogenase 1 and 2 genes and clinical prognosis in AML patients. Furthermore, to evaluate the clinical significance of the SNP in codon105 in the isocitrate dehydrogenase gene1 in AML patients.

Patient material

A retrospective CLL cohort was used in paper I and paper II and consists of 210 patients, diagnosed according to National Cancer Institute-sponsored Working Group (NCI-WG) guidelines criteria for CLL (Hallek et al., 2008). Peripheral blood was collected either at the time of diagnosis or when the patients started the first treatment. The samples were collected between 1996 and 2006 from Linköping University Hospital and mononuclear cells were immediately isolated by Ficoll-Paque gradient centrifugation and stored as cell pellets at -80°C or as viable cells at -196°C until DNA preparation. More information about the patient cohort are found in the papers I and II.

A retrospective AML cohort was used in paper III and paper IV consisting of 207 AML patients from Linköping University Hospital (n=113) and Karolinska University Hospital, Huddinge (n=94) and collected between 1988 and 2010. The patients are diagnosed and grouped in respective risk categories according to the current Swedish AML guidelines and international recommendations, European LeukemiaNet (Döhner et al., 2010). Peripheral blood or bone marrow were collected at the time of diagnosis. After Ficoll-Paque separation the cells were stored either as cell pellets (-80°C) or/and as viable cells (-196°C) until DNA extraction. More details about the patient cohort are found in paper III and IV.

Methods

Some of the methods used in this thesis are described here.

Single strand conformation analysis

In *paper I* and *III* the *TP53* gene and in *paper II* the *NOTCH1/2* genes were screened for mutations by using the single-strand conformation analysis/polymorphism (SSCA/SSCP) first described by Orita et al., 1989. It is a rapid and sensitive method for detection of point mutations used in many laboratories. The first step involves PCR DNA amplification of the fragment of interest. The PCR product is labelled with a radioactive nucleotide (^{32}P -ATP) by a secondary PCR reaction and subsequently denatured by heating to single stranded DNA to maintain formed secondary single strand DNA conformation. The DNA strands are separated on a non-denaturing polyacrylamide gel and a single base alteration can be detected on the altered mobility compared to the wildtype fragments. The sensitivity for base substitution detection in a DNA fragment is dependent on many parameters, where the size of the DNA-strand is one. An optimal sensitivity for a DNA-strand is 150-300 nucleotides. Samples containing only wildtype DNA result in two bands, while samples containing both wildtype and mutant DNA will result in four bands based on the conformation on just what a single nucleotide creates. Further, to determine the exact nucleotide change, the samples of the mobility shift band must be sequenced.

To further increase the sensitivity to detect the mutations, we used one more gel, a MDE (Mutation Detection Enhancement) gel, a polyacrylamide-like matrix that has a high sensitivity to DNA conformational differences. The MDE gel may be used with same SSCA technique as the standard polyacrylamide gels. However, no more mutations were found with the MDE gel compared with the standard gel.

Single nucleotide detection

In *paper I* we used MALDI-TOF MS (Matrix assisted laser desorption/ionization time-of-flight mass spectrometry) to determine the different genotypes in the MDM2 SNP309. This technique is well described in paper I. In short, the PCR product anneals upstream the adjacent polymorphic site and along with the extension primer and the potential dideoxynucleotides (ddNTPs) at this SNP. The DNA-polymerase extension incorporates the corresponding ddNTP and terminates the reaction and depending on which nucleotide is incorporated the extended primer will have different weight and time of flight after excitation by a laser. The different genotypes can thus be determined with the MALDI-TOF MS depending to the different nucleotide mass (**Figure 8**).

Figure 8. The basic principal of SNP genotyping and primer extension with MALDI-TOF MS. (Adapted from Sauer, 2005).

Since the MALDI-TOF MS method was time consuming and the measurements were manually performed, as it was difficult to get it automated, we used another method for genotyping in paper III.

In *paper III* the MDM2 SNP309 genotypes were determined with pyrosequencing technique, suitable for short DNA sequences (Ronaghi et al., 1998). Three primers were designed, one forward, one reverse biotin labelled in the 5' end and the third primer was a sequencing primer for the amplified region. The PCR products are incubated with Streptavidin Sepharose, which binds to the biotin labelled strand followed by denaturation of the strands. By a washing process the PCR product will become single stranded, with only the biotinylated strand bound to the beads, subsequently followed by annealing with the sequencing primer and the four different nucleotides are added stepwise. The method is based on an enzymatic reaction, as when every time a nucleotide is incorporated in the complementary DNA strand, pyrophosphate is released (PPi) and the emitted light is detected by a CCD camera and displayed in a pyrogram. The light intensity is proportional to the number of incorporated nucleotides.

IGHV gene status detection

In *paper I and II* the IGHV gene mutational status is performed by PCR amplification of genomic DNA by using gene specific primer pairs (Van Dongen et al., 2003). The positive PCR products are sequenced and to determine the IGHV identity, the sequence is aligned against germline sequences by using the IMGT/V-QUEST database. More than or equal to 98% identity to corresponding germline sequence is classified as an unmutated IGHV gene.

Statistical analysis

In *paper I-IV*, univariate analyses are carried out with log rank test to compare overall survival between different genotypes or to compare between mutated/wildtype genes. The survival is presented with Kaplan-Meier curves calculated from diagnosis until date of the last follow-up, death or date of allo-SCT. To calculate hazard ratios, Cox proportional hazard model (Cox-regression) is used in *paper I-III* to check for potential confounding and interaction. The results from Cox regression analysis are presented with Hazard Ratios and 95% confidence intervals. To compare genotype distribution between patients with CR or no CR in *paper III and IV* Fisher's exact test or χ^2 are used. The Mann-Whitney or Kruskal Wallis test are used to compare age distribution between genotype groups in *paper III*. P-value < 0.05 is considered significant. In *paper I and II* the statistical analyses are performed using STATA v10.1 and in *paper III and IV* the analyses are carried out using IBM SPSS Statistics v.20.

Results and Discussion

Paper I and paper III

Since the MDM2 SNP309 was first described in 2004 numerous studies have been published in a variety of different cancers (Bond et al., 2007). It is of interest to study this polymorphism as it is located in the promoter region of the MDM2 gene and the first study showed higher MDM2 mRNA and protein levels with the T >G exchange in the MDM2 intron 1 regulating region.

In *paper I* we investigated the prognostic value of MDM2 SNP309 in CLL patients. Our results showed a shorter overall survival for patients with the SNP G allele compared to the wildtype T-allele. It was most pronounced for patients with the G-allele in presence of either mutated *TP53* or unmutated IGHV gene compared to each factor alone. As the median age at diagnosis is high among CLL patients and there is a risk to die in other diseases than CLL, we investigated the influence of the different genotypes in patients younger than the median age at CLL-diagnosis. The result was more pronounced for the groups with the younger patients as in the entire group. Lahiri et al., 2007 was the first group to study the MDM2 SNP309 in CLL both alone and in relation to other SNPs, however they could not find any association with other SNPs or prognostic markers in CLL. In a study by Gryshchenko et al., 2008, they used two independent cohorts (n=140, n=111). They could show that MDM2 SNP309 G-allele was associated with a poor outcome and reduced treatment free survival in similarity with our results, although we did not find any association on time to treatment. Further, two other studies by Zenz et al., 2008 and Kaderi et al., 2010 are in conflict with our results. These research groups found no correlation with the SNP309 G-allele in overall survival and treatment free survival in CLL. There is no obvious explanation for the conflicting results, but differences in the patient cohorts may contribute. Our patient group indicate that these patients had a more aggressive CLL disease, indicated by a higher proportion of patients with unmutated IGHV gene and Binet stage B and C compared with others (Zenz et al., 2008a, Kaderi et al., 2010). Like other research groups who have studied the SNP309 we found the age of onset similar irrespective the genotypes.

In the same study we also analyzed for *TP53* mutations. We found mutations in 6.2% in exon 5-8, however unfortunately our cohort lacks information on 17pdel, which abolish further analysis of the *TP53* locus. We showed a strong correlation with worse overall survival and *TP53* mutations compared to patients with wildtype *TP53* gene. The time difference between diagnosis and the time to first treatment can be long for some CLL patients with the “watch and wait” treatment strategy. For one patient with *TP53* mutation the overall survival was as long as twelve years, however for this patient there was a difference in 8.5 years between diagnosis and sampling day, indicating that the *TP53* mutation occurred during the disease progression. *TP53* mutations are found without 17pdel in about 4-5% independently of each other associated with the same negative impact on prognosis as for 17pdel alone (Zenz et al., 2008b, Dicker et al., 2009, Rossi et al., 2009).

There are now recommendations from the European Research Initiative of CLL (ERIC) (Pospisilova et al., 2012) and the last Swedish guidelines (2013-01-17) to perform *TP53* mutation analysis in the clinical routine before treatment initiation. Today, it seems that deletion or mutation in the *TP53* gene is the most reliable prognostic factor for the treatment decisions in CLL (Sellner et al., 2012), however no optimal treatment are available for patients with *TP53* aberrations/mutations. The most valuable treatment to induce long-term survival today is allo-SCT for patients with impaired *TP53* function, but this is most available for younger patients (Schetelig et al., 2008).

In *paper III* we investigated the same MDM2 SNP309 as in *paper I* but in an AML cohort. In this study we found a significant difference in overall survival between being homozygous for the wildtype TT alleles and harboring at least one G allele in non-normal karyotype patients with wildtype *TP53*. Patients carrying at least one G-allele and wildtype *TP53* had a significantly reduced mean OS compared to wildtype TT patients, 13 vs 29 months. Only a limited number of studies are performed with regard to the SNP309 in AML cells. One is in therapy related AML (t-AML) arisen from prior cytotoxic therapy and this group did not find any significant association between SNP309 and t-AML (Ellis et al., 2008). Another study report a 3.5 increased risk for the G allele associated with AML, however this group did not report any time to first treatment or overall survival data (Xiong et al., 2009).

In *paper III* we also performed a *TP53* gene mutation analysis for exon 5-8. In 207 AML patients we detected 21 (10.1%) patients with a mutated *TP53* gene. Almost all mutations were located to the high risk group AML with non-normal karyotype, 18/83 (21.7%) mostly with complex karyotype and for two patients carrying *TP53* mutations the karyotype data was missing. Patients with *TP53* mutations regardless of the risk group showed a dramatically impaired outcome. In similarity to Grossmann et al. 2012 we showed that no *TP53* mutated patient survived for 3 years. For patients with *TP53* mutations, 78% did not achieve complete remission (CR) compared with 24% for patients with wildtype *TP53*, who did not achieve CR ($p<0.001$). Age at diagnosis was higher for patients with mutated *TP53* than for patients with wildtype *TP53*. Older patients have commonly higher frequency of high-risk cytogenetics, which may have developed through a long period of genetic alterations and thus a more adverse prognosis. *TP53* mutation analysis is not performed in routine analysis, but it would deliver an important prognostic information in AML.

Taken together, *papers I and III*, which study an impaired p53 function reveal that *TP53* mutations and 17pdel are strong prognostic markers in both CLL and in particular AML. Mutated *TP53* is associated with poor response to DNA damaging therapies as the p53 levels are reduced and thus inhibit cell growth or DNA repair or initiate apoptosis are impaired followed by adverse overall survival for patients with *TP53* abnormalities.

The MDM2 SNP309 with at least one G-allele with a negative influence seemed to be most pronounced in AML in the high risk cytogenetically group and in a group with more aggressive CLL where the G-allele may suppress the p53 activity.

For future treatment, the leukemia patients with mutated *TP53* gene, may be treated with drugs restoring the mutated p53, such as PRIMA1 (p53 Reactivation and Induction of Massive Apoptosis) (Bykov et al., 2002, Nahi et al., 2006, Lehmann et al., 2012). Upregulating of wildtype p53 by nutlins, small molecular inhibitors of MDM2, or RITA, which likely inhibits the p53-MDM2 interaction, may represent other future possibilities. (Kojima et al., 2005, Issaeva et al., 2004).

Paper II

In *Paper II* we have investigated the mutational activation of *NOTCH1* and *NOTCH2* genes in CLL, as Notch1 and Notch2 play a critical role in lineage differentiation of white blood cells.

In our study we screened for mutations in the extracellular region, heterodimerisation domain and the PEST domain in *NOTCH1* gene and heterodimerisation domain and the PEST domain of the *NOTCH2* gene. We found *NOTCH1* mutations in 14/209 patients (6.7%). Almost all (13/14) mutations correspond to a 2-bp CT frameshift deletion (c.7541_7542delCT) and one was a 2-bp GT frameshift deletion (c.6988_6989delGT) in the C terminal PEST region, generating premature stop codons and a Notch1 truncated protein. This leads to removal of the PEST amino acid sequence and impaired Notch1 ubiquitin mediated degradation. This results in an intracellular accumulation of truncated Notch1 protein and increased transcription of Notch1 regulated genes. Our analysis showed a shorter overall survival among CLL with *NOTCH1* mutation compared to the Notch1 wildtype patients (p=0.049). We also found *NOTCH1* mutations in a higher frequency together with the unmutated IGHV gene (10/14). Further, patients with *NOTCH1* mutation at diagnosis were more often classified in Binet stage B or C indicating that patients with *NOTCH1* mutation have a more aggressive disease course. *NOTCH1* gene mutations are almost mutually exclusive with the *TP53* gene mutation and only in one (1/14) sample we found both *NOTCH1* mutation and *TP53* mutation. Thus, mutations in the *NOTCH1* or the *TP53* gene represent 12.9% of the cases in our cohort, both resulting in significantly shorter OS compared to wildtype genes. The mutations seem to be localized to two hot spot regions, one in the heterodimerisation region and one in the C-terminal part that eliminates the PEST sequence.

Since, the *NOTCH2* gene has a similar nucleotide structure and protein domain organisation to *NOTCH1* and overlap in some cellular functions, we screened for mutations in the corresponding parts of the *NOTCH2*. However no mutations were found in these regions on the *NOTCH2* gene.

Several studies have been performed with *NOTCH1* mutations analysis displaying the same results as ours with significantly shorter OS with mutated *NOTCH1* gene compared with the wildtype gene. Most mutations reported are a 2 base pair deletion in exon 34 resulting in a frame shift mutation and a premature stop codon removing the critical PEST sequence. Mutation frequencies vary between different studies and are in range of 4.7% - 12.2% (Puente et al., 2011, Fabbri et al., 2011, Rossi et al., 2012a, Mansouri et al., 2013, Willander et al., 2013).

NOTCH1 mutations are reported to be significantly more common with trisomy 12 than any other chromosomal aberration in CLL (Baletti et al., 2012, Del Giudice et al., 2012). In our study no significant association with trisomy 12 was found, in agreement with Mansouri et al., 2013. CLL patients with *NOTCH1* mutations confer high risk of transformation to Richter syndrome, however the molecular mechanisms behind this are unknown (Fabbri et al., 2011 Rossi et al., 2012d). Activation of Notch1 induces activation of e.g. MYC and NF- κ B signaling pathway promoting survival, proliferation and angiogenesis, which may be important for the transformation to diffuse large B-cell lymphoma in CLL. Thus, *NOTCH1* mutations may classify a subgroup of CLL patients with risk for transformation into aggressive lymphoma, Richter syndrome, with decreased OS and poor treatment response (Villamor et al., 2013).

Paper IV

In *Paper IV*, we examined the frequency of mutations in the *IDH1* and *IDH2* genes and whether the mutations correlate to the outcome in AML. In 45% of the de novo AML cases no chromosomal aberrations are detected and those tumors are classified as CN-AML into the intermediate risk group. However, this risk group is heterogeneous, where some patients reach complete remission while others rapidly relapse. Mutations in the *NPM1* gene and *FLT3-ITD* are incorporated as prognostic markers, but there is still a large group of the intermediate risk patients without any reliable prognostic marker for treatment decisions. Mardis et al. (2009) was the first group to investigate *IDH1* mutations in AML and found *IDH1* mutations strongly associated with normal cytogenetic. Several mutation studies have been investigated in both the *IDH1* and *IDH2* genes, but still the clinical impact is unclear.

Our mutation analysis showed *IDH1* to be mutated codon R132 in 16/207 (7.7%) of the patients resulting in four different amino acid exchanges, (R132C, R132H, R132G and R132L). Further, *IDH2* mutations were found in 13% of the patients, where 21/207 (10.1%) were in codon 140 (R140Q and R140G) and 6/207 (2.9%) in codon 172 (R172K). Our results showed that *IDH2* mutations in the intermediate risk patient group with cytogenetic normal karyotype had a significant impact on OS with adverse outcome compared with patients carrying the wildtype *IDH2* gene ($p=0.009$). The mean OS was 3 months for patients with mutated *IDH2* and 21 months for patients with wildtype *IDH2*. No association in CR or OS were evident for patient with *IDH1* mutations in any of the risk groups. Additionally, *IDH1* and *IDH2* mutations are mutually exclusive. No other studies have previously reported any adverse impact on overall survival for patients with *IDH2* mutations in contrast to Ward et al., 2010 and Green et al., 2011 who reported a trend toward improved survival. However, studies have reported high levels of the 2-HG metabolite in *IDH* mutant AML cells (Gross et al., 2010). Further, other studies have demonstrated the possibility to target the 2-HG metabolite and inhibit the 2-HG accumulation and get the AML cells to differentiate (Wang et al., 2013).

We also investigated a synonymous SNP in codon 105 (rs11554137) in the *IDH1* gene with a change from a C to T allele, revealed a significant reduced OS compared to patients with the wildtype C allele in the intermediate CN-AML risk group and negative *FLT3-ITD* ($p=0.003$). The frequency of the SNP was 21/207 (10.1%) in the entire group, very similar to the normal healthy population frequency (11.7%). We observed no correlation between the *IDH1* mutation in codon 132 and the SNP variant in codon 105 although they are close to each other. Analysis

will be required to explain the mechanism behind the SNP in codon 105 in the *IDH1* gene, as the biologic effect of the silent SNP is not elucidated. A German research group found a similar association of the T-allele and decreased survival in AML (Wagner et al., 2010). They showed a higher expression of IDH1 mRNA level for AML patients with the SNP than the wildtype and their hypothesis was that the higher level of the IDH1 protein may affect the chemosensitivity. Additional, this SNP is also found with adverse outcome in patients with malignant gliomas (Wang et al., 2013). A potential mechanism of the synonymous SNP in codon 105 with a replacement of one glycine (GGC) to another glycine (GGT) may possibly, resulting in a new splice variant and introduction of a frameshift and a premature termination and a loss of a part of the coding sequence of exon 4. The consequence of this may be an unstable mRNA and altered IDH1 activity (Kimchi-Sarfaty et al., 2007).

Concluding remarks

In this thesis, genetic alterations have been investigated in chronic lymphocytic leukemia and acute myeloid leukemia.

In CLL, we show that patients with *TP53* mutations had a shorter overall survival and is an independent prognostic marker. DNA-damaging chemotherapeutic agents, such as alkylators and purine analogs are the primary drugs in treatment of CLL and their efficacy is partly dependent on intact *TP53* gene. So far, the only prognostic marker that influences the treatment decision is a dysfunctional *TP53* that is associated with refractory CLL. Deletion in the 17p has been performed with FISH in routine diagnostics, however it is now also recommended to perform *TP53* mutation analysis. The frequency of *TP53* mutations is low at diagnosis and may change over time by mutations and clonal evolution. Therefore it is important to perform *TP53* status analysis before starting treatment as the *TP53* status will guide the choice of treatment. *NOTCH1* mutations in CLL are also suggested to be associated with refractory CLL and have recently been identified in exon sequencing efforts of CLL. In our study, we showed a shorter overall survival for patients with *NOTCH1* mutations. It may be of prognostic value to analyze CLL patients with *NOTCH1* mutations as a subset of the patients with *NOTCH1* mutations will transform into aggressive lymphoma. *NOTCH1* mutations were almost mutually exclusive with *TP53* mutations and combine these two mutations form 12.9% with either *NOTCH1* or *TP53* mutations in CLL patients, may be considered in treatment decisions. With new insight in the deregulated Notch1 pathway, promising inhibitors targeting the deregulated Notch1 pathway for new drugs could be possible.

Mutations in the *TP53* gene is an independent prognostic marker, also in AML and show the most unfavorable outcome, OS was less than 12 months for 20 patients and less than 30 months for one patient. Our studies show that *TP53* gene mutations add prognostic information in addition to 17pdel, which is one of the clinical biomarkers used today. Only four tumors with 17pdel overlap with identified 21 tumors with *TP53* mutations, suggesting that mutation analysis of the *TP53* gene in clinical diagnosis is justified. Prognostic information as *TP53* mutation status may aid in decisions of therapeutic interventions, e.g. bone marrow transplantation or drugs restoring p53 function, in both CLL and AML.

SNPs in the genome may contribute to individual susceptibility and predisposition to disease but also response to drugs or therapies. The MDM2 SNP309 may contribute to attenuate the p53 pathway. In the CLL and AML cohorts, we observed a shorter overall survival for patients with the SNP309 G-allele together with a wildtype *TP53* gene. This effect was limited to the karyotypic non-normal AML subgroup and indicates that when a proper p53 function in the cell is needed, a MDM2 mediated p53 suppression may contribute to tumor progression. Although the SNP309 *per se* not predisposed to leukemia, it may add prognostic information on the p53 function and patients may benefit from drugs targeting MDM2 e.g. nutlins.

Since *IDH* mutations were discovered in AML by Mardis et al. 2009 the role of these mutations have been intensively studied and associated with epigenetic alterations such as CIMP. Our study showed that 21.3% of the AML patients had *IDH* mutations, the majority in the CN-AML subgroup. Further, our result showed that patients with mutations in codon 140 in the *IDH2* gene had a poorer OS than the corresponding wildtype ($p=0.005$) in the intermediate risk group.

Thus, mutations of the *IDH2* gene like the SNP in codon 105 in the *IDH1* gene may be useful as prognostic markers in the heterogeneous intermediate risk group, where other prognostic biomarkers are lacking today. Drugs against the 2-HG oncometabolite resulting in inhibition of 2-HG, thus reduce proliferation and promote differentiation of the AML blasts.

Populärvetenskaplig sammanfattning

I Sverige insjuknar årligen ca 1000 individer i leukemi, som är cancer i de blodbildande cellerna i benmärgen. Leukemi förekommer i alla åldrar, men är vanligast bland den äldre befolkningen. Beroende på cellens ursprung och natur kan leukemi delas in i fyra huvudtyper; akut myeloisk leukemi (AML), akut lymfatisk leukemi (ALL), kronisk myeloisk leukemi (KML) och kronisk lymfatisk leukemi (KLL). Akut leukemi karaktäriseras av omogna celler, som har blockerats i cellutvecklingen. I kroniska leukemier stoppas cellutvecklingen i ett mera moget stadium. Leukemicellerna förökar sig snabbt och återfinns förutom i benmärgen i perifera organ såsom blod och lymfkörtlar där de tränger undan de normala vita blodcellerna, som inte kan fungera, vilket innebär att vitala funktioner inte kan vidmakthållas.

Fokus för denna avhandling har varit att studera genetiska variationer och förvärvade mutationer i tumörceller från KLL och AML patienter och att undersöka hur dessa variationer och mutationer påverkar behandling och överlevnad och att dessa avvikelser kan komma att användas som kliniska markörer för prognos och individanpassad behandling.

Tumörsuppressorn p53 är ett protein, som normalt ska förhindra en cell att omvandlas till tumörcell genom att låta cellen dö (apoptos) eller att reparera cellens muterade DNA. Genen för p53 är ofta muterad i cancerceller och därmed har cellens skyddsmekanism satts ur spel. Bland leukemipatienter är p53 mutationer inte lika frekvent förekommande vid diagnos som för patienter med solida tumörer. I denna avhandling har det studerats hur muterat p53 protein i KLL- och AML-tumörceller påverkar patientens behandling och överlevnad. Resultaten visade att det finns en stark korrelation mellan defekt p53 funktion och dålig överlevnad till följd av ett dåligt behandlingssvar jämfört med patienter, som har normal p53 funktion. Speciellt påtagligt var detta hos AML patienter där ingen patient med defekt p53 överlevde mer än 3 år.

P53 kontrolleras av onkoproteinet MDM2, som har en nyckelroll att negativt reglera p53, genom att p53 uppreglerar MDM2, en negativ feedback-loop bildas, som kontrollerar aktiviteten av både p53 och MDM2. För höga nivåer av MDM2 proteinet försvagar p53 signaleringsvägen. I promotorregionen, som reglerar genen för MDM2, förekommer i befolkningen en naturlig variation i DNA-sekvensen där en DNA-nukleotid är utbytt mot en annan nukleotid (T→G) och där nukleotiden G ger upphov till högre nivåer av MDM2 proteinet än nukleotiden T och därmed försvagar p53. I föreliggande avhandling studerades MDM2 variationen i både KLL- och AML-tumörceller. Resultaten visade att patienter med G-varianten i MDM2 genen hade sämre överlevnad i vissa patientgrupper jämfört med patienter, som bar T-varianten. Denna försämring i överlevnad beror troligtvis på att G-varianten av MDM2 proteinet ger upphov till en lägre p53-nivå, vilket kan resultera i ett sämre behandlingssvar. Vi fann ingen koppling mellan de olika MDM2-varianterna och leukemi.

Vidare studerades en mutation i KLL-tumörcellen i genen, som kodar för proteinet Notch1, vilken har en viktig funktion i att kontrollera cellutmognad, celledelning och cellöverlevnad i blodbildande celler. Olika studier har visat att *NOTCH1* mutationer förekommer i ca 5-12% hos KLL-patienter. I studien i denna avhandling uppvisade ca 7 % av patienterna *NOTCH1* mutationer. *NOTCH* mutationerna ger upphov till att Notch-proteinet inte kan degraderas utan är konstant påslaget och troligtvis aktiveras gener, som styr celledelning. Denna studie likt andra

studier visade att KLL-patienter med *NOTCH1* mutationer har sämre överlevnad än patienter med avsaknad av *NOTCH1* mutation.

I ytterligare ett arbete med AML-celler, studerades mutationer i gener, som kodar för två enzym, vilka ingår i citronsyracykeln. Mutationer i gener, som kodar för enzymerna isocitratdehydrogenas 1 och 2 (*IDH1/2*) har nyligen visats ha betydelse genom att mutationerna medför en blockering av den normala blodcellsutvecklingen. Resultaten i denna avhandling visade att *IDH* mutationer förekom hos ca 21 % av AML patienterna. Vidare visade studien att AML-patienter med *IDH2* mutationer hade sämre överlevnad än patienter med frånvaro av *IDH2* mutationen.

Sammantaget visar denna avhandling på resultat, som kan bidra med nya potentiella prognostiska markörer för att selektera patienter, som kan dra nytta av nya framtida läkemedel, som är riktade mot specifika signalvägar för patienter med KLL och AML.

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