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PROGNOSTIC AND BIOLOGICAL IMPLICATIONS OF EPIGENETIC CHANGES IN LEUKEMIA

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To Marion, Jens, Sofia and Benjamin

ABSTRACT

The field of epigenetic research in hematology and oncology is rapidly expanding. Even so, reliable data linking epigenetic changes to clinical outcomes are scarce. We conducted two retrospective studies in AML. The first (paper I) was performed in 107 AML patients without a previous history of MDS where we approximated the global DNA 5-methylcytosine content with a methylation sensitive restriction enzyme assay, the promoter DNA methylation status of three known tumor suppressor genes, CDKN2B (p15), HIC1 and CDH1, and in a subset of 20 patients genome-wide promoter methylation by the Illumina HumanMethylation27 array. Promoter methylation of CDKN2B was common (66%), and associated with better overall and disease free survival in uni- and multivariate analysis. Average genome wide promoter methylation levels were also associated with overall and disease free survival and correlated inversely with global 5-methylcytosine content, which in turn associated with response to induction therapy. The second study (paper II) was restricted to cytogenetically normal de-novo AML cases. In a test group of 58 samples we investigated genome wide promoter methylation by the Illumina HumanMethylation27 array and correlated the methylation patterns with the mutational status of NPM1, FLT3, CEBPA, IDH1, IDH2, DNMT3A and clinical parameters. We found increased promoter methylation in NPM1 and IDH mutated samples with specific methylation patterns for these two mutations. Compared with a control group of normal myeloid progenitor cells from 9 donors the most differentially methylated genes in AML were those that in previous studies were targeted by Polycomb group proteins in embryonic tissue. Furthermore, we found that the methylation levels of the Polycomb targeted genes were associated with overall and progression free survival. The prognostic association was confirmed in a validation cohort of 60 patients and retained significance in multivariate analysis. The third study of this thesis (paper III) was designed to search for the second tumor suppressor gene commonly thought to reside on chromosome 11q21-23 in CLL, based on the finding of two microdeletions in a previous study. Through DNA methylation screening we found a 48% prevalence of aberrant promoter methylation of the shared two-directional promoter of BTG4 / microRNA-34b/c. Functional studies with stress incubation of primary CLL samples as well as the HG3 cell line showed an selective up-regulation of miR-34b/c transcripts in unmethylated cells, but no induction of BTG4 regardless of methylation status. Chromatin immunoprecipitation experiments showed the presence of repressive chromatin marks in both CLL and normal lymphocytes, which may explain our observation that the basal expression levels of miR-34b/c were low both in normal lymphocytes and CLL cells regardless of methylation status, compatible with a "epigenetic switch" from conditional to permanent silencing in methylated samples.

We conclude that DNA methylation patterns are associated with mutational status and clinical outcomes in AML. Furthermore we believe that miR-34b/c may function as a tumor suppressor gene in CLL, incapacitated by an epigenetic switch mechanism in approximately 50% of CLL samples.

LIST OF PUBLICATIONS

I. **Deneberg S**, Grövdal M, Karimi M, Jansson M, Nahi H, Corbacioglu A, Gaidzik V, Döhner K, Paul C, Ekström TJ, Hellström-Lindberg E, Lehmann S. Gene-specific and global methylation patterns predict outcome in patients with acute myeloid leukemia. *Leukemia*. 2010 May;24(5):932-41.

II. **Deneberg S**, Guardiola P, Lennartsson A, Qu Y, Gaidzik V, Blanchet O, Karimi M, Bengtzén S, Nahi H, Uggla B, Tidefelt U, Höglund M, Paul C, Ekwall K, Döhner K, Lehmann S. Prognostic DNA methylation patterns in cytogenetically normal acute myeloid leukemia are predefined by stem cell chromatin marks. *Blood*. 2011 Nov 17;118(20):5573-82.

III. **Deneberg S**, Kanduri M, Bengtzen S, Karimi M, Qu Y, Kimby, E, Rosenquist R, Lennartsson A and Lehmann S. MicroRNA-34b/c is aberrantly hypermethylated by an epigenetic switch mechanism in B-cell Chronic Lymphatic Leukemia. *Manuscript*

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LIST OF ABBREVIATIONS

AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATM	ataxia telangiectasia mutated (gene)
BM	bone marrow
CDH	cadherin (gene)
CDKN2B	cyclin dependent kinase inhibitor 2b (gene)
CEBPA	CCAAT enhancing binding protein alpha
ChIP	chromatin immunoprecipitation
CLL	chronic lymphocytic leukemia
CMP	common myeloid progenitor
CN	cytogenetically normal
CpG	cytosine-guanine dinucleotide
CR	complete remission
DA	daunorubicin cytarabin (treatment)
DFS	disease free survival
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
FAB	French American British
FLT3	fms like tyrosine kinase 3 (gene)
GMP	granulocyte macrophage progenitor
HDAC	histone deacetylase
HIC1	hypermethylated in cancer 1 (gene)
Hox	homeobox
IDH	isocitrate dehydrogenase (gene)
IGVH	immunoglobulin heavy chain variant region
ITD	internal tandem duplication
LUMA	luminometric assay
MDS	myelodysplastic syndrome
miRNA/miR	micro RNA
MLL	mixed lineage leukemia (gene)
MPD	myeloproliferative disease
MS-MCA	methylation specific melting curve analysis
MSI	microsatellite instability
nc RNA	non coding RNA
NPM1	nucleophosmin 1 (gene)
ORF	open reading frame
OS	overall survival
p15	cyclin dependent kinase inhibitor 2b (alternative gene name)
PcG	polycomb group (protein)
PCR	polymerase chain reaction
PFS	progression free survival
RNA	ribonucleic acid
SCT	stem cell transplantation
TET	ten eleven translocation (gene)
TKD	tyrosine kinase domain
TP53	tumor protein p53 (gene)
UTR	untranslated region

1 INTRODUCTION

1.1 EPIGENETICS-A BRIEF HISTORY

1.1.1 The early theories

The early history of what we today call epigenetic research is inseparable from the history of developmental processes and genetics. Conrad Hal Waddington (1905-1975), professor of Genetics at the University of Edingburgh, was the person who coined the term “epigenetics”, a synthesis of “epigenesis”, i.e the process of organism development, and “genetics”, in an effort to connect these two separate fields of research. He used the term with this denotation for the first time in two publications in 1941 and 1942 [1, 2]. Several researchers contemporary with Waddington, such as Julian Huxley, J.B.S Haldane and Ernst Hadorn, explored the relationship between development and genetics, but they all had different opinions of the definition and used the term “epigenetics” differently[3]. Waddington also introduced the concepts of the “epigenetic landscape” and “canalization”, a model of explaining differentiation and cellular development that still is topical[4]. He developed these concepts based on the observations that there is an innate “robustness” of phenotypes; that nature was able to

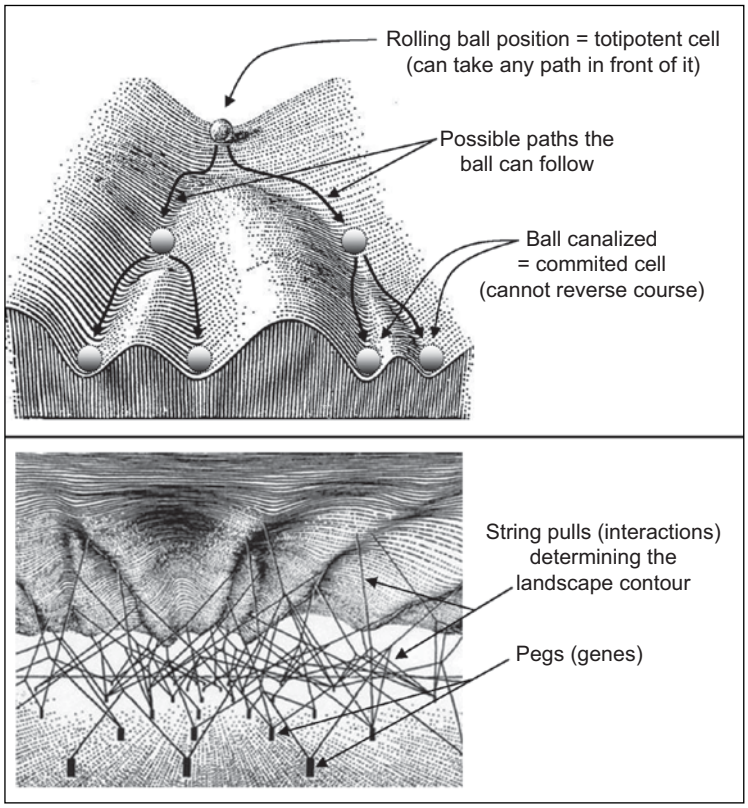

Figure 1 The epigenetic landscape as described by C.H Waddington. The contour of the epigenetic landscape on the upper panel decides which canal the ball will follow. The network strings and poles in the lower panel defines the shape of the landscape in itself. Figures reprinted from the original publication with permission from Taylor and Francis.

buffer the majority of induced genetic variations and environmental exposures, which would have little or no effect on the final phenotype. This concept was called “canalization”, elegantly illustrated in figure 1. The shape of the surface, the epigenetic

landscape, is determined by elevations and valleys forming paths, or canals, in the landscape. If an imaginary ball would be placed on the highest point of the landscape, it would follow the most sloping canal down to the lowest point it could reach, which would be the final phenotype in a developmental system. The model can just as well be applied to cell differentiation, for instance hematopoiesis, where the starting point at "high ground" would equal the hematopoietic stem cell which subsequently differentiates through progenitor and increasingly differentiated stages to reach its final phenotype. Under the surface of the epigenetic landscape it is connected by multiple strings (epigenetic interactions) to firmly attached poles (genes), shaping the landscape above. Changing or removing individual genes (poles) or interactions (strings) would surely affect the shape of the landscape, but since the canals are formed by many poles and strings, there is an inherent robustness in the system.
These early concepts of epigenetics did not provide any insights of specific *mechanisms*, they were rather describing a phenomenon necessary to explain developmental observations – bridging the gap between stable genotype and variable phenotype.

1.1.2 The discovery of epigenetic mechanisms

The existence of 5-methylcytosine in DNA was reported in 1948 [5] by Hotchkiss and confirmed by Wyatt two years later[6]. It took several decades until this discovery was associated with decreased gene transcription, which was first reported in 1975 independently by three groups[7-9]. Riggs suggested a model of X-chromosome inactivation by DNA methylation and Holliday and Pugh that specific gene DNA methylation could explain the switching on and off of genes during development. Also, the existence of maintenance DNA methyl transferases that were sequence specific in themselves or bound to sequence specific proteins, were predicted. This was later confirmed in 1983 by the characterisation of DNA methyl transferase 1 (DNMT1) – the DNA methyl transferase responsible for maintenance methylation[10] and the *de novo* methyl transferases DNMT 3a and 3b in 1998[11]. The discovery and exploration of covalent histone modifications began long before its transcriptional consequences were known, mainly in the context of protein biochemistry. The earliest modifications, discovered in the 1960:s were histone acetylation and methylation[12]. More work on acetylation and its association with transcription was performed in the seventies[13], but the breakthrough and general acceptance of the link between histone tail acetylation and that this preceded and allowed for transcription to occur was not until the late 1980:s and beginning of the 1990:s[14-17].

1.2 SPECIFIC EPIGENETIC MECHANISMS

1.2.1 Cytosine methylation

DNA methylation is a covalent modification of the cytosine ring at the 5´position typically occurring at a CpG dinucleotide. Recently, methylation of the 3´position has been described, but the consequences of 3-cytosine methylation are less explored [18]. In this thesis the term "DNA methylation" refers to 5-cytosine methylation if not otherwise indicated. The DNA methyltransferases are present at the replication fork during S-phase and DNA methylation is thus a replication dependent event[19]. CpG

dinucleotides are scattered across the genome but less common than would be expected by chance, a phenomenon attributed to the spontaneous deamination of cytosine to thymidine in CpG residues which has occurred during evolution[20]. Preferably in the promoter region of genes however, the CpG residues cluster together and form so called “CpG islands”, of typically 800-1200 base pairs length. They can be found in approximately half of all protein coding genes and in contrast to scattered CpG residues, the CpG dinucleotides in CpG islands are typically unmethylated[21]. The promoter regions are sites of transcriptional regulation and transcriptional silencing of DNA through DNA methylation is thought to be achieved by two direct mechanisms; recruitment of transcriptional repressors and interfering with DNA binding of transcriptional activators. Specific methyl CpG binding proteins, the MeCP;s, complex closely with DNA at multiple (MeCP1) or sparsely (MeCP2) methylated CpG sites regardless of DNA sequence[22, 23]. MeCP2 interacts with a co-repressor complex of proteins, including histone deacetylaces (HDACs) and histone methyltransferases (HMTs), reinforcing repressive chromatin state through cross talk between the DNA methylation system and the histone modification system[24]. Recently, focus has been drawn on the mechanism of 5-methylcytosine to 5-hydroxymethylcytosine conversion by the TET enzymes described more detailed in section *1.3.3.7*. The occurrence of 5-cytosine hydroxymethylation is thought to be very common but the exact function of this modified base is still under investigation. It is thought to be involved in DNA demethylation or gene expression regulation, however, the lack of a technique that can accurately and sensitively detect 5-hydroxymethylcytosine has hindered researchers to further explore its potential biological function. This results from the fact that 5-hydroxymethylcytosine behaves very similar to its precursor 5-methylcytosine during bisulfite conversion and restriction enzyme digestion which for are used in most investigations of the subject, including in this thesis[25, 26].

1.2.2 Histone modifications

The nuclear chromatin is a complex of DNA and histones organized as repetitive elements of so called “nucleosomes”. A nucleosome is composed of the histone octamer and the DNA that wraps around it in a left handed supercoil, 150 bp per nucleosome (Figure 2). The histone octamer consists of two molecules each of H2A, H2B, H3 and H4. Histone H1 links the adjacent histones together along with the “linker DNA”. The N-termini of the core histones protrudes from the nucleosome and is called the “tail”. Most, but not all, covalent histone modifications occur in the tail, all facilitated by specific enzymes or protein complexes.

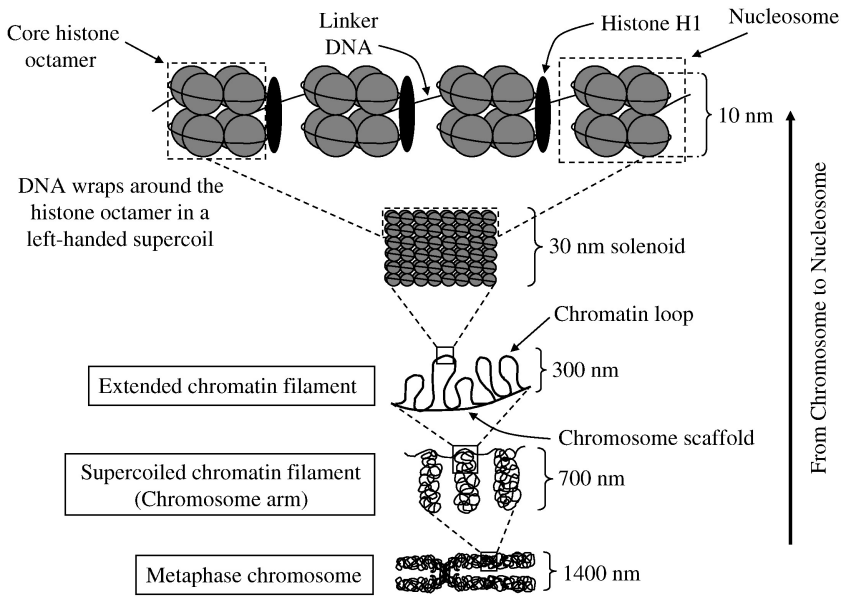

Figure 2 Organisation of chromatin, from methaphase chromosomes (bottom) to nucleosomes (top). Reprinted with permission from Elsevier. Choudhuri et al. Toxicol Appl Pharmacol. 2010 Jun 15;245(3):378-93.

There are different kinds of histone modifications; acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation. The effect they have on transcription is not always clear cut since it sometimes is context dependent. Histone methylation can for instance have both activating and repressing effects depending on at which amino acid the methyl group is deployed, but also depending on how many methyl residues are attached to that particular amino acid. For instance mono or tri-metylation of Lysine 27 at histone 3 (H3K27), the former associated with active and the latter with silent chromatin. A simplified scheme is shown in table 1.
These complex, interacting histone modifications are referred to as the “histone code”, a term that was invented by Strahl and Allis[27, 28] to describe the phenomenon. The term has gained widespread acceptance and is to be understood analogous to the digits on a code-lock; only a specific combination of digits will open the lock. In analogy, only specific histone modification combinations will open the chromatin for transcription.

1.2.3 Micro RNA (miRNA)

Following initial observations in plants of transcriptional inhibition by antisense RNA[29-31]. Craig C. Mello and Andrew Fire's 1998 Nature paper reported a potent gene silencing effect after injecting double stranded RNA into C. elegans. They were the first to recognize the mechanism behind, awarding them the Nobel prize in 2006[32]. They demonstrated the existence of a mechanism where double-stranded RNA successfully silenced the targeted gene and named the phenomenon RNA interference. The endogenous counterpart, micro-RNA:s were initially described in C. elegans in 1993[33], initially thought to be an idiosyncrasy, but subsequent work could show it was a highly conserved feature in multiple species[34,

35]. The majority of miRNA genes are intergenic or oriented antisense to neighboring genes and are transcribed independently[36-38].

Activating modifications	Repressing modifications
Acetylation	
H2A: K5, K9, K13	
H2B: K5, K12, K15, K20	
H3: K9, K14, K18, K23, K56	
H4: K5, K8, K13, K16	
Methylation	Methylation
H3: K4, K36, K79	H3: K9, K27
H3: R17, R23	H4: K20
H4: R3	
Phosphorylation	
H3: T3	
H3: S10, S28	
H3: Y41	
H2AX: S139 (for DNA repair)	
Ubiquitination	Ubiquitination
H2B: K120,	H2A: K119
H2B: K123 (yeast)	
	Sumoylation
	H2A: K126 (yeast)
	H2B: K6, K7 (yeast)
	H4: K5, K8, K12, K16, K20

Table 1 Some histone modification with their effect on transcription. Reprinted with permission form Elsevier from Choudhuri et al. Toxicol Appl Pharmacol. 2010 Jun 15;245(3):378-93.

They are encoded by single pri-miRNA sequences, containing from one to six miRNA precursors. Transcribed by DNA polymerase II these hairpin loop structures are composed of about 70 nucleotides each. The biogenesis is schematically illustrated in figure 3. The double-stranded RNA structure of the hairpins in a pri-miRNA is recognized by a nuclear protein "microprocessor" complex; Drosha-DGCR8, which processes the pri-miRNA to pre-MIRs[39]. Pre-MIRs have a two-nucleotide overhang at its 3' end and have 3' hydroxyl and 5' phosphate groups. These are exported to the cytoplasm and further processed by Dicer, an RNAse II enzyme, resulting in an imperfect miRNA:miRNA duplex of about 22 nucleotides in length[40, 41]. The mature miRNAs are incorporated into an active RNA-induced silencing complex (RISC) containing Dicer, Argonaut2 and many associated proteins[42].

Using the miRNA as a guide, the RISC complex induces specific genetic silencing, either via mRNA degradation or by preventing mRNA from being translated. It has been demonstrated that if there is complete complementation between the miRNA and target mRNA sequence, Arogonaut2 can cleave the mRNA and lead to direct mRNA degradation. Yet, if there isn't complete complementation the silencing is achieved by

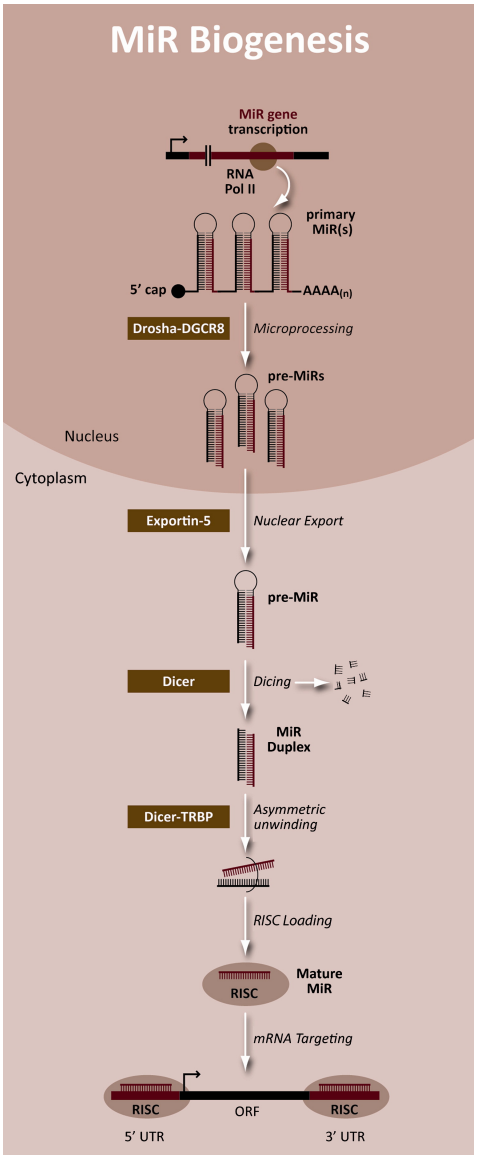

Figure 3 The biogenesis and processing steps of micro-RNA:s, highlighting the processing of primary miR:s by Drosha to pre-miR:s, nuclear export and Dicer processing, loading to the RISC-complex which, using the miR as a targeting device finds the implicated mRNA ORF, resulting in translation inhibition and/or mRNA degradation. RNAPolII=RNA polymerase II. DGCR8=Di George Syndrome Critical Region 8 gene. TRBP= transactivating response RNA-binding protein. RISC= RNA-induced silencing complex. ORF=Open reading frame. UTR=Untranslated region. Reprinted with permission from Elsevier.

preventing translation[42-44]. In humans about 1000 miRNAs have been described[45], and there is amounting evidence of miRNA dysregulation in cancer[46-48].

1.2.4 Long non coding RNA:s (ncRNAs)

In contrast to micro RNA:s there is an abundance of non coding RNA transcripts longer than 200 nucleotides, on the basis of a convenient practical cut-off in RNA purification protocols that excludes small RNA A recent study found only one fifth of the transcription across the human genome is associated with protein-coding genes [49], indicating at least four-times more long non-coding than coding RNA sequences. Large-scale sequencing projects such as FANTOM (Functional Annotation of

Mammalian cDNA) further reveal the complexity of this transcription[50]. The FANTOM3 project identified approximately 35000 non-coding transcripts from 10000 distinct loci that bear many signatures of mRNAs, including 5'capping, splicing and poly-adenylation, but have little or no open reading frames (ORF). They are typically transcribed by DNA polymerase III. Many of these are thought to be functional, however, until now only a relatively small proportion has been demonstrated to be biologically relevant[51, 52]. NcRNAs can target different aspects the of RNA transcription machinery, functioning as co-regulators, modifying transcription factor activity, or regulating the association and activity of other co-regulators or even targeting RNA polymerase II[53-55]. An example is formation of RNA-double helix DNA association, resulting in triple helix formation which has been shown to block the binding site for transtcriptional co-activator TFIID in the promoter of the dihydrofolate reductase gene[56]. Intriguingly, tens of thousands of similar triple helix structures exists throughout the genome[57]. Another type of ncRNAs are the short interspersed nuclear Alu-repeats, which are extremely abundant in the vertebrate genome, comprising approximately 10% of all DNA[58, 59]. These elements can be transcribed as ncRNAs as a response to stress, for instance heat shock, and bind to RNA polymerase II, inhibiting the formation of the pre-initiation complex[60-62]. Non coding RNAs also interfere with other epigenetic regulation. An example is HOTAIR, a ncRNA encoded from the HOXC locus which when expressed represses part of the HOXD locus by directing the Polycomb Repression Complex II to this locus, inducing histone trimethylation[63, 64]. Another example is the function of ncRNA XIST, which during embryonic stem cell differentiation is expressed from the X-chromosome that will be inactivated. It coats the X-chromosome and induces repressive chromatin modifications; H3K27me3, H3K9 hypermethylation and H4K20 monomethylation, H2AK119 monoubiquitylation and H4 hypoacetylation, resultion in inactivation[65, 66]. In cancer the world of ncRNAs is still largely unexplored, but some studies have been performed. For instance, a recent study observed an inverse expression profile of the p15 gene and an antisense ncRNA (CDKN2BAS) in AML, correlating the degree of p15 DNA methylation with the level of antisense ncRNA expression[67].

1.3 ACUTE MYELOID LEUKEMIA

1.3.1 Overview, definition and pathogenesis

Acute Myeloid Leukemia (AML) is the predominant type of acute leukemia in adults. It is a disease characterized by clonal expansion of immature hematopoetic cells of the myeloid lineage, myeloblasts, of more than 20% in the bone marrow[68], however, the presence of certain chromosomal rearrangements are diagnostic with lower blast counts. The disease affects approximately 4/100 000 persons yearly in the western world which amounts to 3-400 cases yearly in Sweden[69, 70]. The median age at diagnosis is 71 years[71]. The classical symtoms are those of bone marrow failure, i.e. infections due to leukopenia, fatigue and breathlessness due to anemia and bleeding/bruising due to thrombocytopenia. The etiology is unknown, although preceding malignancies such as myelodysplastic syndromes (MDS) or myeloproliferative disease (MPD) are not uncommon. There is also an association with exposure to ionizing radiation and benzene as well as previous chemotherapy [72, 73]. The classification of AML has traditionally been based on morphologic features ever since the first French-American-British (FAB) classification of 1976[74, 75]. In an

effort to incorporate the growing body pathophysiological and genetic knowledge, the first WHO classification came in 2001 and was revised in 2008[68, 76, 77]. The WHO classifications took account of cytogenetic findings, relation to previous chemotherapy as well as the presence of dysplastic lesions. Cases that were not defined by these criteria fell under the "AML, not otherwise specified" group, which essentially consists of the old FAB classes (table 2).

AML med vissa specifika genetiska aberrationer
t(8;21)(q22;q22)[1]; *RUNX1-RUNX1T1*[1]
inv(16)(p13;q22) eller t(16;16)(p13;q22); *CBFB-MYH11*[1]
t(15;17)(q22;q21); *PML-RARA*[1]
t(9;11)(p22;q23); *MLLT3-MLL*
t(6;9)(p23;q34); *DDEK-NUP214*
inv(3)(q21;q26.2) eller t/3;3)(q21;q26.2); *RPN1-EVI1*
t(1;22)(p13;q13); *RBM15-MKL1*
Provisorisk entitiet: AML med muterad NPMI
Provisorisk entitet: AML med muterad CEBPA

AML relaterad till MDS ("AML related to myelodysplasia related changes")
− Transformation av tidigare känd MDS eller MDS/MPD och/eller
− Dysplasi föreligger i > 50% av celler i åtminstone 2 linjer och/eller
− "MDS-liknande" cytogenetik[2]

Terapi-relaterad AML (AML-T)

Myelosarcom ("myeloid sarcoma")

Myeloid proliferation relaterad till Downs syndrom[3]
− "Transient abnormal myelopoeisis"
− "Myeloid leukemia associated with Downs syndrome"

"Blastic Plasmacytoid Dendritic Neoplams"[3]

Övriga AML	
Inklusionskriterier för någon av ovanstående grupper ej uppfyllda. Följande subkategorier definieras med ytterligare kriterier	
a	Akut myeloblastleukemi med minimal differentiering (FAB M0)
b	Akut myeloblastleukemi utan utmognad (FAB M1)
c	Akut myeloblastleukemi med utmognad (FAB M2)
d	Akut myelomonocytleukemi (FAB M4)
e	Akut monoblast- och akut monocytleukemi (FAB M5 a+b)
f	Akut erytroid leukemi (FAB M6)
g	Akut megakaryoblastleukemi (FAB M7)
h	Akut basofil leukemi
i	Akut panmyelos med myelofibros

Akuta leukemier med oviss linjetillhörighet
− Akut odifferentierad leukemi (uttryck av HLA-DR, CD34, CD38, ev TdT och CD7)
− "Mixed phenotype" akut leukemi med t(9;22)(q34;q11.2); *BRR-ABL1*
− "Mixed phenotype" akut leukemi med t(v;11)(v;q23); *MLL*-rearrangemang
− "Mixed phenotype" akut leukemi, B/myeloid
− "Mixed phenotype" akut leukemi, T/myeloid
− *Provisorisk entitet: "natural killer (NK)-cell lymphoblastic leukemia/lymphoma"*[3]

Fotnot		
[1]	Leukemidiagnos kan ställas även när andelen blaster är < 20%.	
[2]	Cytogenetiska avvikelser vars förekomst berättigar till subdiagnos "MDS-relaterad AML" ("AML with myelodysplasia related changes") förtutsatt ≥ 20% blaster i blod eller märg.	
	Undantag	I de fall AML-sjukdomen är relaterad till tidigare mutagen behandling hän-förs den till subgruppen "terapi-relaterad AML"
		-7 eller del(7q)
		-5 eller del(5q)
		i(17q) eller t(17p)
		-13 eller del(13q)
		del(11q)
		del (12p) eller t(12p)
		del(9q)
		idic(X)(q13)
		t(11;16)(q23;p13.3)
		t(3;21)(q26.2;q22.1)
		t(1;3)(p36.3;q21.1)
		t(2;11)(p21;q23)
		t(5;12)(q33;p13)
		t(5;7)(q33;q11.2)
		t(5;17)(q33;p13)
		tt(5;10)(q33;q21)
		t(3;5)(q25;q35)
[3]	Handläggning av patienter med denna subtyp av AML diskuteras ej närmare i dessa riktlinjer. Angående AML hos patienter med Downs syndrom hänvisas till pediatriskt vårdprogram (www.nopho.org)	

Table 2 The WHO classification of Acute Myeloid Leukemias 2008

1.3.1.1 Genetics

Genetically, AML is one of the most studied types of neoplasms and among the first where genetic information was incorporated into clinical decision making[78]. Recurrent non random translocations, inversions and deletions are typically seen in all hematological malignancies including AML, for instance t(8;21), inv(16) and t(15;17)[79]. Furthermore, molecular aberrations such as nucleophosmin 1 (NPM1) mutations, fms-like tyrosine kinase 3-internal tandem duplication (FLT3-ITD), CCAT/enhancer-binding protein alpha (CEBPA) and Mixed Lineage Leukemia (MLL) translocations have in recent years contributed to prognostic stratification[80] and also, in the case of FLT3-ITD, efforts of using targeted therapy[81].

1.3.1.2 Class I and II mutations

Given the recurrent chromosomal aberrations and molecular mutations it was suggested that AML is the consequence of two complementary classes of mutations: class I that confer a proliferative advantage by activating signal transduction pathways in hematopoetic/myeloid progenitors, and class II that impair differentiation and/or confer properties of self-renewal by affecting transcription factors or transcriptional activation[82, 83]. Mutations of either class are often reciprocal, but this is not universally found[84]. The recently discovered common mutations in AML, DNMT3A and IDH1 and IDH2, TET2 as well as splicing machinery (i.e SF3B1) mutations have not yet found their established place as class I or class II mutations[85-90], figure 4. Interestingly, DNMT3A, IDH and TET2 mutations all impact epigenetic aberrations or mechanisms. Also, which class the mutations affecting the p53 apoptotic pathway belongs to is also debatable, and some authors even place it in a separate class[84, 91].

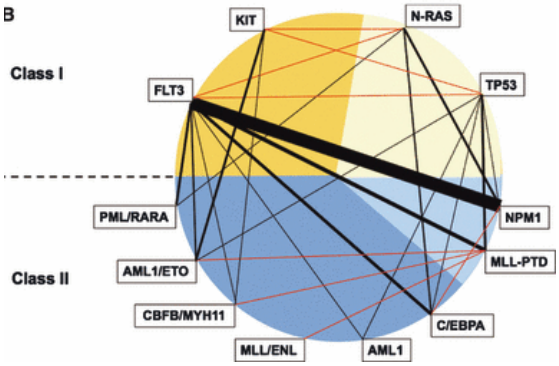

Figure 4 A graphic view of class I and class II mutations. Black lines indicate concurrent mutations in samples, the with of the line indicates frequency among 144 patients. Red lines indicate concurrent mutations of the same class. Reprinted with permission from Wiley. European Journal of Haematology Volume 83, Issue 2, pages 90-98, 21 mar 2009

1.3.2 Prognostic factors

AML is a heterogeneous disease and despite advances in therapy and supportive care prognosis remains poor. Even for patients younger than 65 there is a clear-cut effect of age on survival (figure 5), with median survival at five years of 40%. Among patients older than 65, a dismal 20% are alive at five years after diagnosis[71, 92]. Age is an independent prognostic factor although the inability to cope with intensive regimens, adverse cytogenetics and poor performance status all increase in prevalence with age[93-97]. Even so, some elderly patients seem to benefit from intensive induction[71].

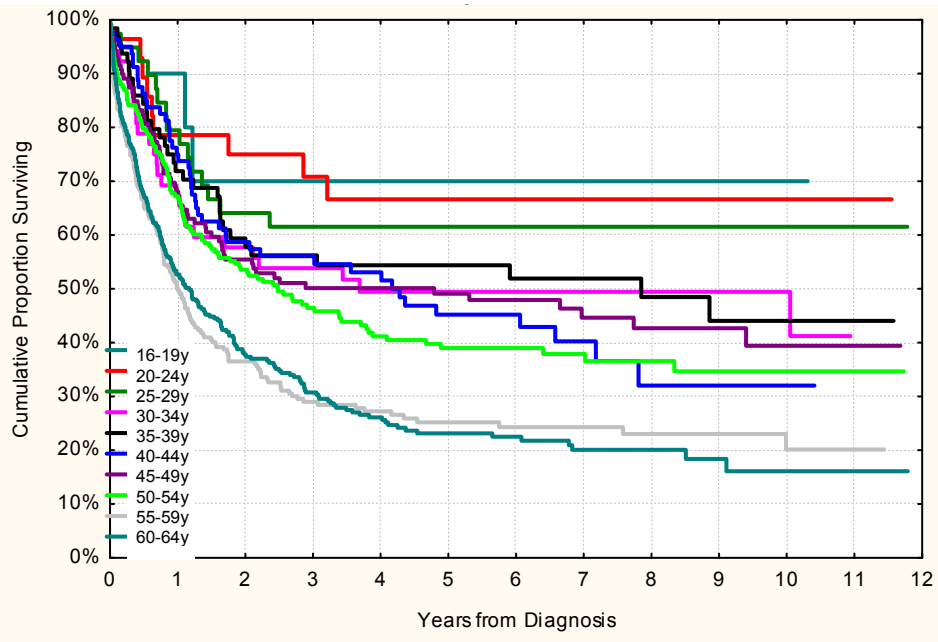

Figure 5 Survival of different age cohorts in patients up to 64 years of age. Data from the Swedish AML registry 2010, showing a continuous decrease in survival with a possible threshold at 55 years. Reprinted with permission.

Genetic group	Subsets
Favorable	t(8;21)(q22;q22); RUNX1-RUNX1T1
	inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	Mutated NPM1 without FLT3-ITD (normal karyotype)
	Mutated CEBPA (normal karyotype)
Intermediate-I*	Mutated NPM1 and FLT3-ITD (normal karyotype)
	Wild-type NPM1 and FLT3-ITD (normal karyotype)
	Wild-type NPM1 without FLT3-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); MLLT3-MLL
	Cytogenetic abnormalities not classified as favorable or adverse†
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
	t(6;9)(p23;q34); DEK-NUP214
	t(v;11)(v;q23); MLL rearranged
	−5 or del(5q); −7; abnl(17p); complex karyotype‡

Table 3 Cytogenetic and molecular risk groups. Adopted from European Leukemia Net 2010[98].

* Includes all AMLs with normal karyotype except for those included in the favorable subgroup; most of these cases are associated with poor prognosis, but they should be reported separately because of the potential different response to treatment.

† For most abnormalities, adequate numbers have not been studied to draw firm conclusions regarding their prognostic significance.

‡ Three or more chromosome abnormalities in the absence of one of the WHO designated recurring translocations or inversions, that is, t(15;17), t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23), t(6;9), inv(3) or t(3;3); indicate how many complex karyotype cases have involvement of chromosome arms 5q, 7q, and 17p.

The karyotype of the leukemic cells is the strongest prognostic factor for response to induction therapy and for survival[99, 100]. Patients are routinely divided into

favorable, intermediate and adverse risk groups based on cytogenetic findings[79, 101-103], see table 3. The complex karyotype has been defined differently over the years, with ≥5 or ≥3 cytogenetic aberrations. The current definition according to the European Leukemia Net is three or more chromosomal aberrations, excluding the WHO designated translocations or inversions. They are thought of as separate entities, since long recognized for the core binding leukemias; t(8;21), inv(16) as well as for APL t(15;17), but now also including several other (table 4)[75].

Complex karyotype AML is often associated with deletion 17p and/or mutations of TP53[102, 103]. A recently highlighted entity is the "monosomal karyotype", defined as either two autosomal monosomies or a monosomy together with at least one additional autosomal aberration, the presence of which infers an even worse outcome than the traditional complex karyotype does[104]. Especially within the group of patients with cytogenetically normal AML, molecular gene mutations of NPM1, FLT3 and CEBPA have been incorporated in the prognostic stratification [78].

1.3.3 Molecular mutations in AML

1.3.3.1 FLT3

FMS-like tyrosine kinase 3 (FLT3) is a member of the class III receptor tyrosine kinase family. It is important in hematopoetic stem cell proliferation, survival and differentiation. Mutations are activating and of two types, affecting either the juxtamembrane domain or the tyrosine kinase domain. The principal juxtamembrane mutation is the FLT3 internal tandem repeat (FLT3-ITD) and the principal tyrosine kinase domain mutation is an activating point mutation (FLT3-TKD)[104-106]. Recently a novel type of FLT3-ITD was discovered, affecting the kinase domain in approximately 30% of FLT3-ITD cases[107]. FLT3-ITD occurs in approximately 20% of all AML cases, somewhat more in cytogenetically normal AML (CN-AML) (28-34%). FLT3-TKD mutations are found in 5-10% of all AML but is more prevalent in inv(16) AML (14-24%)[80]. The clinical prognosis of AML cases with FLT3-ITD is significantly inferior within the CN-AML subgroup and there may be an effect of mutation burden on prognosis[108, 109].

1.3.3.2 NPM1

Nucleophosmin 1 is a chaperone protein shuttling between the nucleus and cytoplasm. It has multiple functions, among them stabilisation of $p14^{Arf}$ in the nucleolus, ribosome biogenesis and export[110-113]. NPM1 mutations are typically heterozygous and mainly affect exon 12 resulting in disruption of the nuclear export signal and accumulation in the cytoplasm[114, 115]. It occurs in one third of all AML cases and around 60% of CN-AML and is frequently associated with FLT3-ITD and IDH mutations[80]. NPM1 mutated AML is a provisional entity in the 2008 WHO revised criteria[77, 116]. NPM1 mutations in the absence of FLT3-ITD has a favourable prognosis, with increased remission rates and better survival among younger patients[80, 115, 117], and a generally better prognosis in elderly[118]. The *European Leukemia Net* recently suggested this entity to be considered as part of the favourable genetic risk category together with core-binding leukemias[98].

1.3.3.3 CEBPA

CCAAT enhancer binding protein alpha (CEBPA) is a transcription factor involved in myeloid differentiation. Mutations of CEBPA occur in 7% of all AML cases but more

often in CN-AML (10-18%). Mutations affect the N-terminal (nonsense mutation) and/or C-terminal (in frame mutations), both giving rise to dysfunctional proteins[119]. In one third of CEBPA mutated cases one allele, and in two thirds both alleles are mutated; 90% having an N-terminal mutation on one and C-terminal on the other allele, called compound heterozygotes[120, 121]. These double mutant cases are associated with a higher CR rate and favourable survival[122-124]. Even transcriptional silencing of CEBPA, notably often by methylation of its distal promoter, has been shown to be of prognostic significance[125, 126].

1.3.3.4 IDH1 and IDH2

Isocitrate dehydrogenase (IDH) 1 and 2 are metabolic enzymes that are rate limiting in the Krebs cycle and are hence strictly regulated. Its physiological function is to convert isocitrate to α-ketoglutarate, but the mutated forms instead cause a gain of function, facilitating production of 2-hydroxyglutatate. The mutations are always heterozygous and IDH1 and IDH2 mutations are more or less mutually exclusive[127, 128]. Both forms are found in approximately 16% of all AML cases, and are somewhat more common in CN-AML (20-37%). They have an unfavourable impact on outcome in the large subgroup of CN-AML with NPM1 mutations lacking FLT3-ITD [129-131]. Interestingly, IDH mutations and TET2 mutations are mutually exclusive and display similar DNA hypermethylation patterns, possibly due to the inhibitory effect of 2-hydroxyglutarate on TET2 function[132].

1.3.3.5 WT1

WT1 is a transcription factor implicated in the regulation of apoptosis, proliferation and differentiation of hematopoetic progenitors[133, 134]. Inactivating mutations occur in 10-13% of CN-AML and have been associated with inferior prognosis[135, 136], but also without prognostic impact[137].

1.3.3.6 MLL

Mixed Lineage Leukemia (MLL) is a DNA binding protein regulating gene expression by its histone methyltransferase activity. The MLL-partial tandem duplication (PTD) mutation is found in approximately 10% of CN-AML and 90% of AML with trisomy 11[138]. MLL-PTD cases are associated with a poor prognosis and a specific DNA methylation profile with up-regulation of homeobox (HOX) genes[139, 140].

1.3.3.7 TET2

The TET2 enzyme belongs to a family of three enzymes. The close relative TET1 converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), and TET2 mutant cases displayed decreased 5hmC-levels. DNA methylation was on the whole not very different, but samples from patients with low 5hmC showed hypomethylation relative to controls at the majority of differentially methylated CpG sites[141].
TET2 mutations occur in approximately 13% of all AML cases, preferably in the intermediate cytogenetic risk group and are more common in isolated trisomy 8 cases[142]. Another group reported a prevalence of 23% in CN-AML cases[143]. Both these studies reported a negative impact on prognosis and were mutually exlusive of IDH mutations.

1.3.3.8 DNMT3A

DNMT3A is one of the *de novo* DNA methyl transferases. It is mutated in approximately 18-23% of AML cases, and is an independent poor prognostic factor for survival[88, 144]. It occurs more frequently in CN-AML cases with IDH, NPM1 and FLT3 mutations. Interestingly, global average DNA methylation levels did not differ from mutated vs. wild type DNMT3A cases, but had different methylation patterns. A

recent study shows that the ability of DNMT3A to form tetramers is impaired by mutations which negatively affects the binding to DNA but not the DNA methylation functions, compatible with disrupted methylation patterns[145].

1.3.4 Epigenetic mechanisms in AML

1.3.4.1 Aberrant DNA methylation

Due to the relative ease of investigating DNA methylation with standard laboratory techniques and the obtainability of AML blasts of relatively high purity there is a huge number of publications on the subject [146, 147]. In contrast with molecular and karyotypical aberrations, there seems to be hundreds of genes aberrantly methylated in AML. Depending on methodology and the number of genes investigated it seems that there are no cases of AML where aberrant DNA methylation is not a feature [148]. In line with this, there is evidence that the levels of DNMT1, DNMT3A and DNMT3B are increased in blast cells of AML patients [149]. Global hypomethylation were among the first described changes in AML [150]. The reason for the apparent contradiction between global hypomethylation and increased gene specific CpG island methylation is an issue that is not resolved. It has been suggested that alternate transcripts of DNMT3B, lacking the catalytic domain may be responsible [151] but other studies counter that [152]. Other explanations may be increased activity of DNA demethylases [153], folate deficiency or the previously predominant view that the global hypomethylation is a passive process. The crosstalk between histone modifications and DNA methylation is also implicated. Loss of monoacetylation of H4Lys16 and trimethylation of H4Lys20 in leukemia cells correlate with DNA hypomethylation of *Sat2*, *NBL2* and *D4Z4* tandem repeat sequences [154]. A recent publication where whole genome bisulfite sequencing was performed in colon cancer and normal controls may help to resolve this issue[155]. Hansen et al describes "blocks" of hypomethylated DNA covering more than half the colon cancer genome that largely overlap with "nuclear lamina-associated domains", i.e. chromosomal regions that associate with the nuclear membrane and are transcriptionally silent[156]. The also describe numerous small (<5kbp) differentially methylated regions (DMR:s) between normal and cancer where the physiological homeostasis of hypomethylation in CpG islands with a loss of or shift of boundary preferably leads to hypermethylation of CpG islands and relative hypomethylation of CpG island shores. A minority of small DMR:s have the opposite characteristics, i.e hypomethylation of CpG islands and hypermethylation of shores.

There is no consensus regarding the connection between global DNA hypomethylation and the alleged pro-neoplastic events coupled with this such as increased chromosome breakage, increased microsatellite instability (MSI) and concurrent defects in the mismatch repair system seen for instance in colon cancer [157]. MSI is however not an uncommon finding in AML. The prevalence of MSI ranges from 20-30% and perhaps even higher in therapy related and secondary AML [158]. The hMLH1 and hMSH2 genes are involved in the DNA mismatch repair (MMR) pathway and are closely associated with MSI. They are silenced by promoter hypermethylation in several neoplasms, but this is a rare event in AML, seen in only 2-4% of cases[159].

Numerous studies have investigated specific gene promoters for methylation in AML and the number of individual genes found hypermethylated is exhaustive[160-168]. An interesting finding is the inverse correlation of age and the number of genes methylated found in one study, perhaps indicative of a different methylation pattern in de novo AML [169]. On a genome-wide scale a few studies have been published as to date showing high levels of CpG island hypermethylation non-randomly distributed. Using

restriction landmark genome scanning (RLGS), Rush et al. could show promoter methylation in 8.3% of CpG islands [170]. No studies have however shown a stable correlation between DNA methylation patterns and the French-American British (FAB) histological AML subtypes with the exception of FAB M3 -Acute Promyelocytic Leukemia (APL) [148]. Recent genome wide methylation analyses have confirmed that different methylation patterns exist among AML cases that can only partially be explained by distinctive molecular and cytogenetic subgroups. Figueroa et al. [148] conducted a large scale DNA profiling study of 344 AML samples showing specific methylation profiles for each of the core binding leukemias, t(8;21), t(15;17) and inv 16 as well as CCAT Enhancer Binding Protein-alpha (CEBPA) mutation and silencing as well as different profiles for NPM1 mutated cases. Cases harboring 11q23 translocations also clustered separately in this study, in line with other studies that have described a specific methylation profile for Mixed Lineage Leukemia (MLL)-translocated AML, also elegantly shown by functional experiments by Alvarez et al. [171]. The histone methyltransferase activity of MLL, a gene with multiple tranlocation partners in 5% of AML, may partially explain this [172, 173]. Interestingly, MLL is also a common target for activating partial tandem mutations (PTD) with an incidence of 5-11% in cytogenetically normal AML [105]. MLL-PTD mutants retain the functional C-terminal SET-domain that has methyltransferase activity. This mutated form of MLL is a marker of poor prognosis and has been shown to up-regulate certain Homeobox genes by an increase of H3/H4 acetylation [174] and is associated with a genome-wide increase in promoter methylation [140]. Furthermore the specific methylation pattern seen in APL, characterized by t(15;17), may be explained by the activity of the fusion protein PML-RARα which interacts with DNMTs and direct them to RARα-targets, causing promoter DNA methylation and gene silencing [175]. The oncoprotein PML-RARα has also been shown to interact with other epigenetically acting proteins such as HDACs and the Polycomb repressive complex 2 [176, 177]. The first line treatment for APL, pharmacological doses of all-trans-retinoic-acid (ATRA), dissociates the repressive complexes from the promoter, inducing re-expression of the silenced genes and concurrent differentiation of the APL cells [175]. Similar mechanisms have also been suggested for another fusion protein, AML1-ETO, the product of t(8;21), that participates in a protein complex with RARα, recruiting DNMTs and HDACs inducing a repressive epigenetic state at these promoter sites [178]. EVI-1, overexpressed in inv(3) AML is associated with unfavorable outcome and has recently been shown to display a specific genome wide DNA methylation signature and interact with DNMT3A and DNMT3B [179]. In other tumor types bivalent histone marks as well as Polycomb Group enriched promoters in embryonic stem cells have predisposed the promoter methylation patterns seen in cancer [180]. There is some evidence that similar mechanisms are active in AML as shown for CDKN2B by Zangenberg and Paul et al. [181, 182]. To summarize, some of the aberrant methylation patterns seen in AML have molecular explanations. However, identification of these as well as identifying which aberrations are driving the disease and which are only “passengers” remains a challenge.

1.3.4.2 Histone modifications

In contrast to DNA methylation histone modifications have been relatively little investigated in AML. On a genome wide level H3K9 trimethylation was investigated in AML patients and found decreased at promoter regions. Interestingly, a H3K9me3 pattern also predicted event free survival in this group of AML patients [183]. There were no apparent correlation between H3K9me3 and known cytogenetic aberrations. An investigation of the relations between activation (H3K9me3) and repressive (H3K27me3) chromatin marks in relationship to DNA methylation of the CDKN2b locus on chromosome 9 found that H3K9me3 was lost in samples with DNA

methylation of the same loci, but both H3K9me3 and H3K27me3 co-existed, even when activating transcription of CDKN2b with 5-aza-2'-deoxycytidine (a DNMT inhibitor) and trichostatin A (an HDAC inhibitor)[184].

1.3.5 Treatment

1.3.5.1 Current standard treatment

Most patients younger than 70 years of age receive intensive chemotherapy aimed at inducing a complete remission, meaning less than 5% leukemic blasts in the bone marrow after recovery after treatment, recovery meaning a neutrophil count of >1.0x10^9/L and a platelet count of >100x10^9/L[185]. Since the 1970:s the backbone of AML-treatment has been a combination of daunorubicin and cytarabine (DA) [186, 187]. Several different combinations and alternative anthracyclines have been tried, none with convincingly better results[188-190], although high dose Daunorubicin (90mg/m^2) seems to be superior to the previous international norm, 45 mg/m^2[191, 192]. After achieving CR two to three DA consolidation treatments are given often including repeated DA courses and/or intermediate/high-dose Ara-C [193, 194]. Consolidation with allogeneic stem cell transplantation (allo SCT) in first remission or after successful treatment after relapse depends on individual risk stratification. Currently all high-risk patients with tolerable co-morbidity below 65-70 years of age are eligible for allo SCT in CR1[195]. Intermediate risk patients most likely also benefit from allo SCT, at least those with a sibling donor, and several centers already practice this for the majority of intermediate risk patients[196-198]. However, the prognostic variability in this patient cohort is wide and there is a need of better prognostic instruments to select for allo SCT[199-202].

1.3.5.2 Epigenetically acting treatment

There is currently tremendous hope for epigenetically acting therapeutic agents in AML, following the landmark analysis that showing a survival benefit of Azacytidine in high risk MDS and AML following MDS [203]. In this study patients were randomized either to treatment with Azacytidine or conventional therapy including high-dose induction or palliative approaches at the treating physicians discretion. There was a survival benefit for Azacytidine treated patients where median survival was 24.5 compared to 16.0 months (p=0.005). Several studies before and after have explored the effects of Decitabine and Azacytidine in elderly with high risk MDS and AML, however often both de novo AML and AML following MDS have been included. Overall response rates have varied from 22% to over 50% with various dosing regimens and combinations [204]. Many efforts have been made to find predictive markers for success of DNMT-inhibitory therapy but so far none has emerged as a stable predictor. There are some indications that demethylating therapy may be more effective in high risk MDS with chromosome 7 abnormalities and/or chromosome 5 abnormalities (excluding del (5q) in MDS)[205, 206], but this finding could not be verified in a large retrospective study recently published [207]. Lower baseline methylation levels have been suggested to vouch for a better effect of Azacytidine [205]. In a recent publication of a phase II trial of Decitabine in older patients with previously untreated AML, responders to therapy had higher baseline values of miR-29b, a micro-RNA that targets DNMT3a and DNMT3b directly and DNMT1 through Sp1 and thus lower the global DNA methylation levels[208]. This view is however challenged in a recent publication that found no evidence that either global methylation

levels or promoter methylation of specific tumor suppressor genes could predict response to Azacytidine in combination with the histone deacetylase inhibitor Entinostat [209].
A multitude of phase I and phase II studies have shown efficacy of DNMT-inhibition in MDS [210, 211]. The two FDA approved agents, 5-azacytidine and 5-aza-2'-deoxycytidine were originally designed as cytosine analogues but later proved to inhibit DNMTs, likely have more modes of action than just pure demethylating activity [212]. Also supporting this is the lack of correlation between demethylation of specific promoters and cell death and that the pattern of gene activation only partially can be explained by promoter demethylation[213, 214]. However, as shown in the genome-wide study by Figueroa et al. there is a gross reduction of promoter methylation already at day 15 after treatment start with 5-azacytidine and entinostat, much earlier than blast clearance is expected [215]. The slow response to treatment is seen both with Decitabine and Azacytidine treatment and does speak against pure cytotoxic effects, which usually manifests itself by rapid blast clearance.
Histone deacetylase inhibitors have also been explored alone or in combination regimens for MDS and AML [216, 217]. Phase I studies of Vorinostat (SAHA) and MGCD0103 showed effect in a population with mainly MDS and AML patients and were well tolerated[216, 218]. As with DNMT inhibitors the mode of action of HDACi is not thought to be entirely dependent of epigenetic mechanisms. Even if gene reactivation has been shown it seems that at least SAHA works through induction of reactive oxygen species [219].
In conclusion, neither DNMT nor HDAC-inhibitors have yet found their place in the treatment of AML with the possible exception of AML following MDS, where it may have merit. However, there is insufficient data to pronounce DNMT-inhibitors superior to induction therapy even in that setting.

1.4 CHRONIC LYMPHOCYTIC LEUKEMIA

1.4.1 Overview and pathogenesis

Chronic lymphocytic leukemia (CLL) is one of the most common leukemias in the western world, characterized by infiltration of morphologically mature small B-lymphocytes in lymphoid tissue, bone-marrow and blood with a typical immunophenotype; CD5+, CD19+, CD23+. The yearly incidence in Sweden is 500, and the median age is 72, with a male predominance of roughly 2:1[220]. The pathogenesis is not completely known, however a genetic component is likely due to the increased incidence in relatives[221]. The CLL cells origins from an antigen.experienced B cell with recombination of the V(D)J genes at the Ig locus. Approximately 50% of CLL cases also have somatically hypermutated IGHV genes, an event that normally takes place in B-cell maturation, perhaps indicating a more differentiated cell of origin, in contrast to the 50% of cases with unmutated IGVH genes[222]. Since CLL cells are slow growing ex vivo interphase FISH has become the method of choice to investigate chromosomal aberrations in CLL. Recurrent aberrations can be found in more than 80% of CLL cases; 13q, 17p and 11q deletions and trisomy 12 are the most common ones. Deletion 13q is found in approximately 50%, deletetion of 11q in 20%, 17p in 7% and trisomy 12 in 12% of CLL cases. In 13q

deleted CLL cases, the minimally deleted region includes miR-15 and miR-16 which were the first micro RNA:s to be shown to be bona-fide tumor suppressor genes in any malignancy[223]. One important mechanism of action of miR-15 and miR-16 is that haploinsuffiency of these miR:s increases the expression of Bcl-2, critical for tumor cell survival[224]. No specific gene/genes have been convincingly implicated as oncogenes on chromosome 12. Deletion of 11q often, but not always, includes the Ataxia Telangiectasia Mutated (ATM) kinase gene and is also coupled to an inferior prognosis[225]. ATM is mutated in 12% of all B-CLL cases and in 30% of del 11q cases, which indicates that there may be other tumor suppressor genes in this region[226]. Deletion 17p encompasses the TP53 gene and is often accompanied by mutation of TP53 on the remaining allele.

1.4.2 Prognostic markers

1.4.2.1 Clinical staging

The Rai and Binet clinical staging systems have been used for more than 30 years. They are based on the presence of absence of lymphadenopathy, splenomegaly and signs of bone marrow failure – anemia and/or thrombocytopenia.

1.4.2.2 Cytogenetics

Cytogenetic analysis by FISH is also an important prognostic tool. Deletion of 13q is considered to be a good prognostic marker. Trisomy 12 is a sign of intermediate prognosis. Deletion 11q has been associated with extensive lymphadenopathy and is a poor prognostic marker. Deletion 17p is a marker of extremely poor prognosis.

1.4.2.3 Mutational status

IGVH mutational status is also a prognostic factor in CLL. A cutoff at 98% sequence homology of IGHVs in CLL with the germline copies of IGHVs is typically used to define the separation. However, this is an arbitrary cutoff, and a grey-zone exists; for instance those that have a IGHV homology of 95–98%) seem to have an intermediate prognosis[227].

1.4.3 Treatment

Chlorambucil is an alkylating agent used since more than 50 years in the treatment of CLL[228]. With the emergence of newer, more effective agents, the use of chlorambucil has declined and nowadays it is usually reserved for elderly patients with comorbidity.

Fludarabine and its family member cladribine are at least as effective as chlorambucil as single agent therapy[229], but is preferably used in combinations, especially since there is an association between fludarabine single agent therapy and severe immune cytopenia[230]. Several combinations of fludarabine have been investigated, and one of the most popular at the moment is the combination with the alkylating agent cyclophosphamide (FC). Compared with single agent fludarabine both response and progression free survival (PFS) was better with the combination, however, overall survival (OS) was not increased[231]. Furthermore the incidence of autoimmune cytopenias was significantly reduced. The humanized monoclonal antibody alemtuzumab is directed at the CD52 antigen, common to normal and malignant B and T cells. Compared to chlorambucil it induces better response and PFS, but not OS[232]. It seems to have equal effect in 17p deleted patients as it has in others and is thus

recommended as first line therapy for 17p deleted cases[233]. It is often used in combination with FC, however that combination seems inferior to the combination of rituximab (anti CD-20) with FC[234], which is the first combination to achieve increased overall survival in CLL[235]. Allogeneic stem cell transplantation remains the only potentially curative treatment in CLL. Use is however limited by toxicity and it is mainly used in younger, fit patients relapsing after standard therapy or TP53 mutations[236].

1.4.4 A p53- micro-RNA- ZAP-70 feedback loop in CLL

Since the discovery of micro-RNA:s two decades ago the understanding of their importance of them in oncogenic transformation and progression has expanded. CLL was the first tumor where micro RNA:s could be shown to play a role via the implication of miR-15 and miR-16 in 13q deleted CLL, which was demonstrated both by association and with functional studies[223]. Subsequently several micro-RNA:s have been shown to be important in other tumors as well as in CLL. A comprehensive overview was recently published in JAMA, where a pathogenetic model of the involvement of micro-RNA:s and protein coding genes was presented (Figure 6)[237]. In CLL samples and cell lines, miR-15 and miR-16 targeted the TP53 gene. In turn, expression of p53 induced both miR-15/miR-16 as well as the miR-34b/c clusters, the latter in turn targeting and down regulating ZAP-70. In a large group of CLL samples levels of p53 were indeed higher in homo or heteroallelic 13q deleted (where the miR-15/16 cluster is located) samples than in samples with normal karyotype. Also, samples with 11q deletions (where the miR-34b/c cluster is located) had lower expression of miR-34b then samples with normal karyotype. Conversely, patients with 11q deletions had significantly higher levels of ZAP70 than patients with CLLs with normal cytogenetic profiles. Interestingly, the miR-34-family has been implicated in several other cancer types, often inactivated by promoter methylation; colon cancer, prostate, lung, ovarian, breast, pancreatic, urothelial and renal cell carcinoma [238-240].

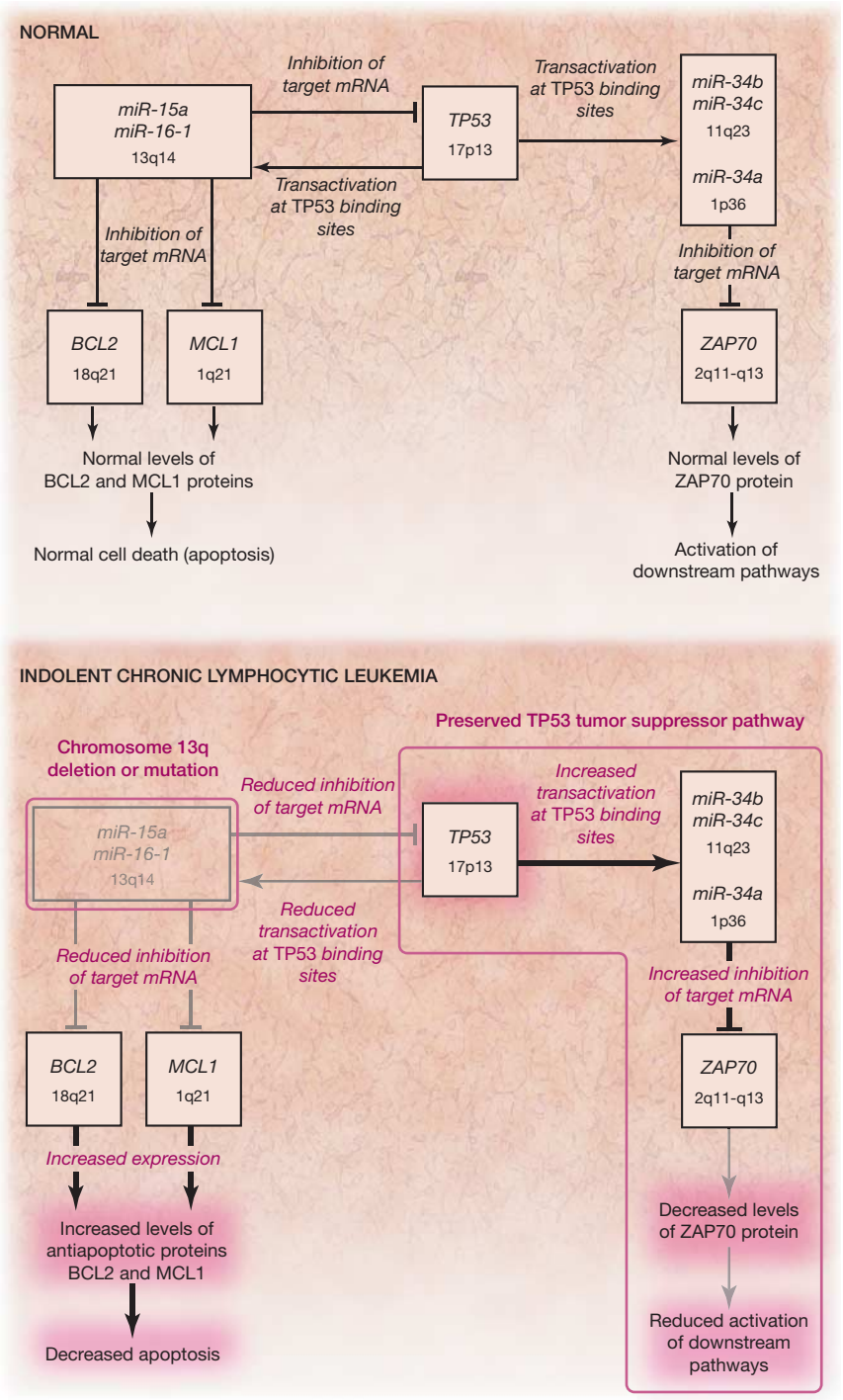

Figure 6 The relationship between TP53 inactivation, the miR-34 and miR-15-16 family, ZAP-70, BCL2 and MCL1 expression in normal lymphocytes (upper panel) and B-CLL (lower panel). Reprinted with permission.

2 AIMS OF THE THESIS

2.1 PAPER I AND II

Principal aim:
To find AML subsets defined by distinct prognostic DNA methylation signatures.
Secondary aims:
To relate these subsets with clinical, cytogenetic and molecular subclasses of AML.
To find novel tumor suppressor candidate genes.

2.2 PAPER III

Principal aim:
To find a second tumor suppressor candidate gene on chromosome 11q23 in CLL apart from ATM.
Secondary aim:
To relate this tumor suppressor candidate gene´s epigenetic status to response to chemotherapy and prognosis in CLL.

3 MATERIAL AND METHODS

3.1 PATIENTS AND TREATMENT

The World Health Organization 2008 criteria were applied [77] for assessing diagnosis and classification of CR and progression. All patients gave informed consent at the time of sampling in accordance with the declaration of Helsinki and the respective local ethics committee approved the studies.

In paper I a total of 107 previously untreated AML patients treated at the Karolinska University Hospital at Huddinge were included. Patients with AML M3 or a previous history of MDS were excluded. Standard induction chemotherapy including cytarabine and an anthracycline was given aiming at CR. Patients who achieved CR were given standard consolidation therapy according to the applicable national or study protocol including allogeneic stem cell transplantation. Of all patients reaching CR, 18 (24%) received allogeneic stem cell transplantation, 41 (54%) received full consolidation therapy and 16 (22%) received reduced chemotherapy consolidation because of treatment toxicities.

In paper II primary bone marrow (BM) samples from 118 CN-AML patients were obtained at diagnosis from patients at the Karolinska University Hospital, Huddinge (n=55), Uppsala University Hospital (n=15), Örebro University Hospital (n=9), Ulm, Germany (n=10) and Angers, France (n=29). Patients with AML M3 or a history of MDS or MPD or previous chemotherapy were excluded. Standard induction chemotherapy including cytarabine and an anthracycline was given aiming at CR. Patients who achieved CR were given standard consolidation therapy according to the applicable national or study protocol including allogeneic stem cell transplantation which was performed in 39 (33%) of the patients.

In paper III peripheral blood from 66 CLL patients from Uppsala University Hospital (n=60) and Karolinska University Hospital, Huddinge (n=6) was collected. Various therapy regimens were delivered as deemed fit by the treating physician. No patient underwent allogeneic stem cell transplantation.

3.2 NORMAL CONTROLS

In paper II, bone marrow from nine healthy controls was collected, all with informed consent in accordance to the declaration of Helsinki and approved by the ethics committee.

3.3 INCUBATION OF CELLS

In paper III CLL and normal cell incubations as well as the HG3 CLL cell line incubations were performed in RPMI Medium 1640 with Glutamax and HEPES buffer (Invitrogen) with 10 % FBS added. 3-5 million cells were incubated for 48 hours in 37°C at a cell density of 300.000 cells/ml in 5μM 5-aza-2-deoxycytidin (Decitabine) and 0.1 μM doxorubicin and 1 μM Prima-1.

3.4 CELL SEPARATION AND STORAGE

All AML samples were separated for mononuclear cells by density gradient centrifuging (Lymphoprep; Axis-Shield PoC). In cases when DNA was not extracted immediately, cells were vital frozen in -150°C. BM from healthy donors (n=9) was separated for mononuclear cells by Lymphoprep (Axis-Shield). BM CD34+ cells were further separated by MACS, indirect CD34 microbead kit (Miltenyi Biotec), according to the manufacturer's instructions. In five cases, the common myeloid progenitor (CMP) and granolucyte- macrophage progenitor (GMP) cells were purified from the CD34+ pool with FACS, using antibodies specified in supplemental Table 4 as previously described[241] and CD34+ cells were used in four cases.

CD38	FITC	
CD110	PE	
CD123	PerCP	Cy5.5
CD33	PE	Cy7
CD34	APC	
CD45RA	Pacific Blue.	
CMP; CD38+, CD34+, CD123+, CD110 , CD45RA+		
GMP; CD38+, CD34+, CD123+, CD110 , CD45RA		

Table 4 Antibodies used to separate CMP and GMP stages in paper II.

CLL cells were collected from peripheral blood from CLL patients and contained >70% lymphocytes according to immunophenotyping. Normal lymphocytes were collected from healthy volunteers and from mixed leukocyte DNA from multiple donors (Roche, Bromma, Sweden). CD19+ normal leukocytes were purchased from 3H Biomedicals (Uppsala, Sweden). CLL and normal cell incubations as well as the HG3 CLL cell line incubations were performed in RPMI Medium 1640 with Glutamax and HEPES buffer (Invitrogen) with 10 % FBS added.

3.5 NUCLEIC ACID SEPARATION

DNA was separated using the DNeasy kit (Qiagen, Valencia, CA, USA) or the QuickGene kit (Fujifilm, Stockholm, Sweden). RNA was extracted using the RNeasy kit (Qiagen) according to instructions, and quality was controlled using an Agilent Bioanalyzer (Agilent Technologies). DNA was stored in -20°C and RNA in -80°C.

3.6 MOLECULAR, IGVH AND FISH ANALYSES

Mutation analyses of FLT3 (internal tandem duplications and tyrosine kinase domain mutations at codon D835 and I836) and NPM1 were performed by the Döhner laboratory in Ulm as previously described[109, 131]. IDH1 and IDH2 (exon 4) were also investigated in Ulm. Briefly, DNA fragments spanning the entire exon 4 of IDH1 and IDH2 were amplified by polymerase chain reaction (PCR) by using Optimase Polymerase (Transgenomic, Glasgow, United Kingdom) and gDNAs as templates. IDH1 and IDH2 amplicons were screened for heterozygous mutations by using denaturing high-performance liquid chromatography (dHPLC) on a WAVE 3500HT

DNA Fragment Analysis System (Transgenomic). The individual dHPLC chromatograms were compared with a wild-type reference; samples that differed from the wild-type reference were reamplified in an independent PCR reaction and were assessed for sequence variations by direct sequencing or sequencing after subcloning. For CEBPA, complementary DNA (cDNA) was generated from 1 μg of mRNA using SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA). The CEBPA coding region was divided into three overlapping amplicons. After PCR amplification, 10 μl of PCR product was mixed with 10 μl of corresponding PCR product obtained from NB4 cell line cDNA. Heteroduplexes were allowed to form in an Applied Biosystems (Foster City, CA) GeneAmp PCR System 9700 (2 cycles of 95°C for 3 minutes, cooled to 220°C with a ramp of 5%, and maintained at 20°C for 5 minutes). The samples were then subjected to dHPLC analysis. Samples with aberrant peaks were subjected to direct nucleotide sequencing on an Applied Biosystems 3100 device using the forward and reverse primers. In case a mutation was found, a second analysis on new input material was performed to rule out PCR-induced artifacts. In AML cases for which dHPLC had revealed one single heterozygous mutation, the CEBPA coding region was fully sequenced to exclude the possibility that a second mutation had gone unnoticed. Detailed protocols including primer sequences are available online[131, 242].
IgHV mutational status was investigated by PCR amplification and sequencing as previously described[243]. Briefly, VH gene family-specific polymerase chain reaction (PCR) amplification was performed as previously described[244]. In the majority of samples clonal PCR products were sequenced directly using the BigDye Terminator Cycle Sequencing Reaction Kit (Perkin-Elmer, ABI, Foster City, CA), but in 13 cases cloning of the PCR products was performed as described elsewhere[244]. The sequences were aligned to IgH sequences from the BLAST, V-BASE, and IMGT databases. VH gene sequences deviating more than 2% from the corresponding germline gene were defined as mutated. The distribution of replacement mutations and silent mutations within the CDRs and framework regions was assessed according to the multinomial distribution model published by Lossos and colleagues to determine the extent of antigen selection in mutated VH genes[245]. The length of the CDR3 was calculated between codon 95 and 102.
Interphase FISH analysis was performed on CLL samples for del(13q) (D13S319, LAMP1), del(11q) (ATM), trisomy 12 (centromere), and del(17p) (TP53) using commercial probes from Abbot Vysis (Stuttgart, Germany) as previously described[225]. Two-hundred interphase nuclei were analyzed for each probe and sample. The cut-off level for samples to have a particular aberration was 10%.

3.7 BISULFITE CONVERSION AND DNA METHYLATION ANALYSES

Bisulfite conversion was performed manually in paper I as previously described[246]. Briefly, 2.5mg of DNA from each sample was denatured in NaOH for 15 min at 37 1C, followed by incubation in sodium bisulfite at 551C for 16h. Thereafter, DNA was recovered using the GeneClean II kit (Qbiogene, Montreal, QC, Canada), desulfonated in NaOH and precipitated in ethanol. The EZ DNA methylation kit (Zymo Research) was used for bisulfite conversion of 500-1000 ng of DNA per sample in papers 2 and 3.

3.7.1 Methylation specific melting curve analysis

The method was developed by professor Guldberg in Copenhagen and is described in detail previously[247]. PCR of the promoter region was performed with SYBR green-containing master mix. Immediately after amplification, a melting curve was produced and compared to a positive (SSS1 treated DNA, Roche) and negative control (Mixed Human Leukocyte DNA, Invitrogen). Samples with right shifted melting curves compared to the negative controls were considered methylated. Primers were designed using the Oligo1.0 software (Molecular Biology Insights, Cascade, CO, USA) for MS-MCA and primer sequences are given in the paper, supplementary table I.

3.7.2 Denaturing gradient gel electrophoresis (DGGE)

The method has been described in detail previously[248]. In brief, the PCR products were loaded on a 10–70% denaturant gradient gel together with a fully methylated control (In vitro methylated DNA) and an unmethylated control (peripheral blood lymphocytes). After electrophoresis, gels were stained in Tris/EDTA buffer containing ethidium bromide and photographed under ultraviolet transillumination. Samples were scored as methylated when bands or smears were present on the gels in the area below the band corresponding to unmethylated DNA as reported in previous publications[248].

3.7.3 Luminometric assay (LUMA)

Global DNA methylation was quantified by luminometric assay (LUMA) as previously described[249, 250]. A total of 500 ng genomic DNA was cleaved with HpaII plus EcoRI or MspI plus EcoRI in separate reactions. After the digestion step, the extent of cleavage was quantified by pyrosequencing. HpaII and MspI are methylation-sensitive isoschizomers. DNA methylation is assessed by the HpaII/MspI ratio.

3.7.4 Bisulfite pyrosequencing

Bisulfite pyrosequencing was performed for the CDKN2A, CDH1, HIC1, and CDKN2B promoter. After bisulfite conversion pyrosequencing was performed on the PyroMark12 platform (QIAGEN) as previously described.12 Primer sequences and PCR conditions are available at: www.techsupport.pyro sequencing.com. Samples were in general considered as methylated at mean levels of more than 15% methylation.

3.7.5 DNA methylation arrays

Genome-wide DNA methylation profiling was performed using the Illumina Infinium HumanMethylation27 BeadChip (Illumina) for 20 samples in paper I and 89 samples in paper II, of which 29 were performed in Angers, France and the rest at the BEA core facility at Novum, Karolinska Institutet. Upon treatment with bisulfite, unmethylated cytosine residues are deaminated to uracil while methylated cytosines remain unaffected. The assay interrogates these differentiated loci using two site specific probes, one designed for methylated residues and one for unmethylated. Single-base extension of the probes incorporates a biotin labelled ddNTP, which is subsequently stained with a fluorescence reagent in order to determine the signal intensities of

methylated vs unmethylated probes. The EZ DNA methylation kit (Zymo Research) was used for bisulfite conversion of 500-1000 ng of DNA, and the remaining assay steps were performed as previously published, using Illumina-supplied reagents and conditions[251]. The readout from the array is a β-value, which is defined as the ratio between the fluorescent signal from the methylated allele to the sum of both methylated and unmethylated allele and thus correlates to the level of DNA methylation. A β-value of 1.0 corresponds to complete methylation and 0 equals no DNA methylation.

3.8 GENE EXPRESSION ANALYSES

Whole genome expression arrays were performed by the BEA core facility at the Karolinska Institute using the Illumina HumanHT-12 Version 4 Expression BeadChip using the Direct Hybridization Assay and iScan system (Illumina). Details of the procedure can be accessed online:
http://www.illumina.com/products/humanht_12_expression_beadchip_kits_v4.ilmn
Quantitative RT-PCR was performed using primers, control genes and PCR conditions as described in each paper.

3.9 CHROMATIN IMMUNOPRECIPITATION

Chromatin Immunoprecipitation (ChIP) in paper III was performed using Magnetic LowCell ChIP kit from Diagenode (Denville, NJ, USA) according to manufacturer's instruction, using antibodies against H3K27me3 (Abcam, ab6002), H2Az (Abcam, ab4174) and H3 (Abcam, ab1791) for normalization as previously described[252].

3.10 STATISTICAL ANALYSES

Categorical clinical parameters and gene-specific variables were analyzed in relation to CR by chi-square analyses or Fisher's exact test as appropriate. In paper I, the contribution of gene promoter methylation and global methylation to DFS and OS were assessed using the Kaplan–Meier method and compared with the log-rank test. Binary logistic regression was used for multivariate analysis of CR. Cox regression analysis was used for multivariate analysis of DFS and OS. Testing for proportional hazards was performed by plotting the log of hazard for each included variable from the Kaplan-Meier curves against time. Correlations were calculated with Pearson's correlation for continuous variables and Spearman's rho for ordinal data. Multiple-group comparisons were made using one-way analysis of variance. In paper II statistical analysis of the methylation array data was carried out in the statistical computing language R (www.r-project.org). To search for the differentially methylated genes between the different prognostic subgroups, the data were arcsin transformed and an empirical Bayes moderated t test was then applied using the "limma" package. The P values were adjusted using the method of Benjamini and Hochberg and a level of P less than 0.05 was used as a cut-off. An additional filter for the average geometric difference was also applied to assure only genes with absolute differences of more than β 0.10 between the groups remained. Integration of gene expression array data with methylation array data and integration of previously published ChIP-chip results were made with the BeadStudio Version 3.2 software (Illumina). Unsupervised hierarchical

clustering analysis and principal components analysis were performed with the Genesis software package 17.1. Hierarchical clustering analysis distance was measured using Pearson correlation with a complete clustering algorithm. Samples were normalized using a global mean centering method. Methylation levels of different molecular subgroups were measured as continuous variables. Hypermethylation of individual CpG sites was defined as β -values more than 0.7, unmethylated as β less than 0.3, and values in between were called "methylated". Differences in overall and progression free survival was evaluated using the Log-Rank test after construction of Kaplan-Meier curves. Multivariable analyses were performed using Cox Regression analysis for survival and Logistic regression for remission rates. In paper III differences in expression between groups were calculated using the Students T-test or the Mann-Whitney test when appropriate and the relationship between promoter methylation and cytogenetic aberrations or IgVH status by the Chi-square test. SPSS versions 16-20 (IBM) were used for statistical calculations.

4 RESULTS AND DISCUSSION

4.1 GLOBAL DNA METHYLATION IS INVERSELY CORRELATED WITH CPG ISLAND METHYLATION

In paper I and paper II samples were investigated with LUMA, which is a surrogate marker for global 5-methylcytosine content. In paper I twenty samples were also investigated with the IlluminaHuman27 methylation bead array, an array with a strong bias towards promoter regions and CpG islands. The relationship of global methylation as measured by LUMA was found to be inversely correlated with the number of hypermethylated CpG sites in the Illumina array (defined as β>50%), Pearson correlation 0.627, P=0.003 (figure 7, upper panel). The results were corroborated in paper II where 29 samples were investigated by both methods and the Pearson correlation was -0.45, P=0.02 (figure 7 lower panel). The reason for the inverse signs of the correlation coefficients was that LUMA methylation was calculated differently in the two publications; HpaII/MspI in paper I and 1-(HpaII/MspI) in paper II.

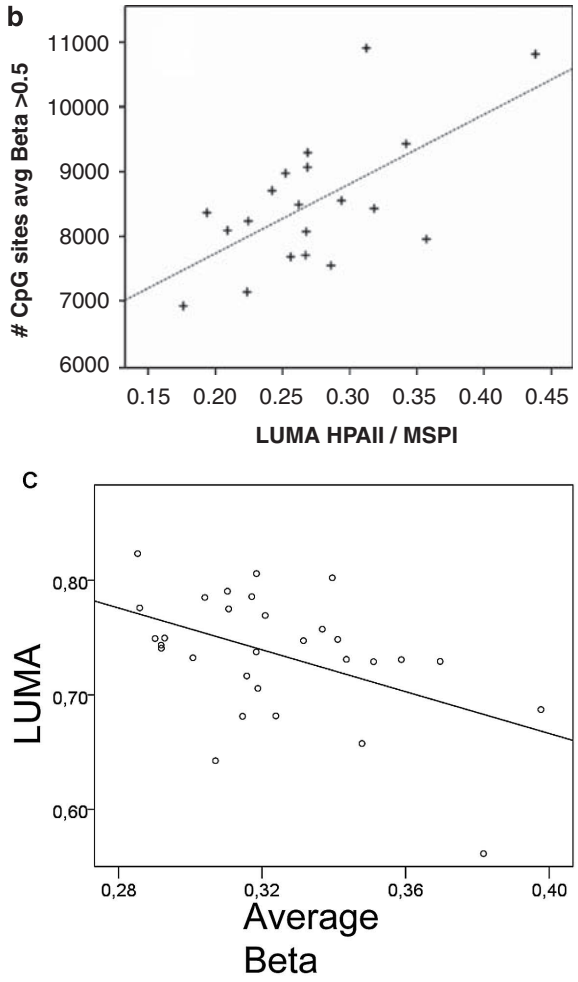

Figure 7 The upper panel shows the relationship between global methylation measured by LUMA and Illumina array methylation for 20 samples from paper I. R=0.627, P=0.003. The lower panel show the same relationship for 29 samples from paper II. R=-0.45, P=0.02. See text for details.

Our interpretation of this is that we have found that in AML, CpG islands in general become methylated while non-CpG-island DNA becomes hypomethylated and that the extent of these changes correlate. This conclusion relies on the validity of our methods. Do we actually measure global, non CpG-island methylation with LUMA? We believe that it does. Since LUMA analysis is based on the HpaII/MspI recognition sequence, 5' -CCGG-3' which are fairly well interspersed through the genome- approximately 50% of the CCGG sites are located in repetitive DNA sequences and 50% in unique sequence[253], and even though they are accumulated at CpG islands, CpG islands only comprise about 1% of the genome[254]. On the contrary, of the 27578 CpG sites on the Illumina HumanMethylation 27k array, 97,7% interrogates CpG sites within 1500 bp from the transcription start site. Even if this finding is novel in AML, the inverse relationship has been described in several other tumors[255, 256]. A recent bisulfite sequencing study of colon cancer found the same phenomenon and also in detail could describe the loss of homeostasis of DNA methylation in cancer compared to normal tissue[155]. The most common finding was disintegration of the boundary between low methylation in CpG islands and high methylation in surrounding DNA, resulting in a smoothening effect with hypomethylation of non CpG island DNA and hypermethylation of CpG islands, occurring in "blocks". Although yet to be verified in AML with bisulfite pyrosequencing, these results are fully compatible with our findings.

4.2 GLOBAL DNA METHYLATION MEASURED BY LUMA CORRELATE WITH CLINICAL OUTCOMES

In paper I we found a borderline significant correlation between low global methylation levels by LUMA and CR rate (P=0.05). In subset analyses, the correlation seemed to be limited to patients younger than 65 years of age (P=0.02), where low global methylation also translated to improved overall and disease free survival (figure 8). The reason for the increased CR rates in patients with global hypomethylation observed in our study is unclear. It is significant in multivariate analysis, but the genes most differentially methylated between patients achieving CR and those not achieving CR are different from the genes governing overall and disease free survival. In paper II, where we had access to genome wide methylation status of a larger cohort, we found that there were several interesting genes among those most significantly associated with CR, for instance; glutathione transferase (GSTP1), a gene central to protection against oxidative stress and xenobiotics as well as implicated in anti-cancer drug resistance[257-259]. Promoter methylation of GSTP1 is an independent factor for increased anthracycline chemosensitivity in breast cancer[260]. MMP-7, a matrix metalloproteinase, has been shown to inhibit anthracyclin induced apoptosis while silencing of MMP-7 with antisense cDNA increased sensitivity[261]. Targeting MMP-7 with inhibitors has proven efficacious in inducing chemosensitivity in various tumor models[262]. DHH[263], LCP-1[264, 265], TRAF-1[266] are other genes implicated in chemoresistance that were found to be significantly more methylated in patients achieving CR and inversely correlated to global methylation by LUMA.
There were somewhat fewer patients with adverse and more with favorable karyotype in the hypomethylated group, but these differences were not significant in multivariate analysis. One explanation of the limitation of the effects to younger patients could be that intra-individual methylation variation accelerates with increasing age, which could

affect both global and gene-specific methylation and hence make cross-sectional analyses such as ours less predictive in an older population[254, 267]. Another explanation may be that AML in older subjects actually is an epigenetically different disease, perhaps more similar to MDS where disrupted DNA methylation patterns in general is a degenerative phenomenon and a bad prognostic sign[166, 215].

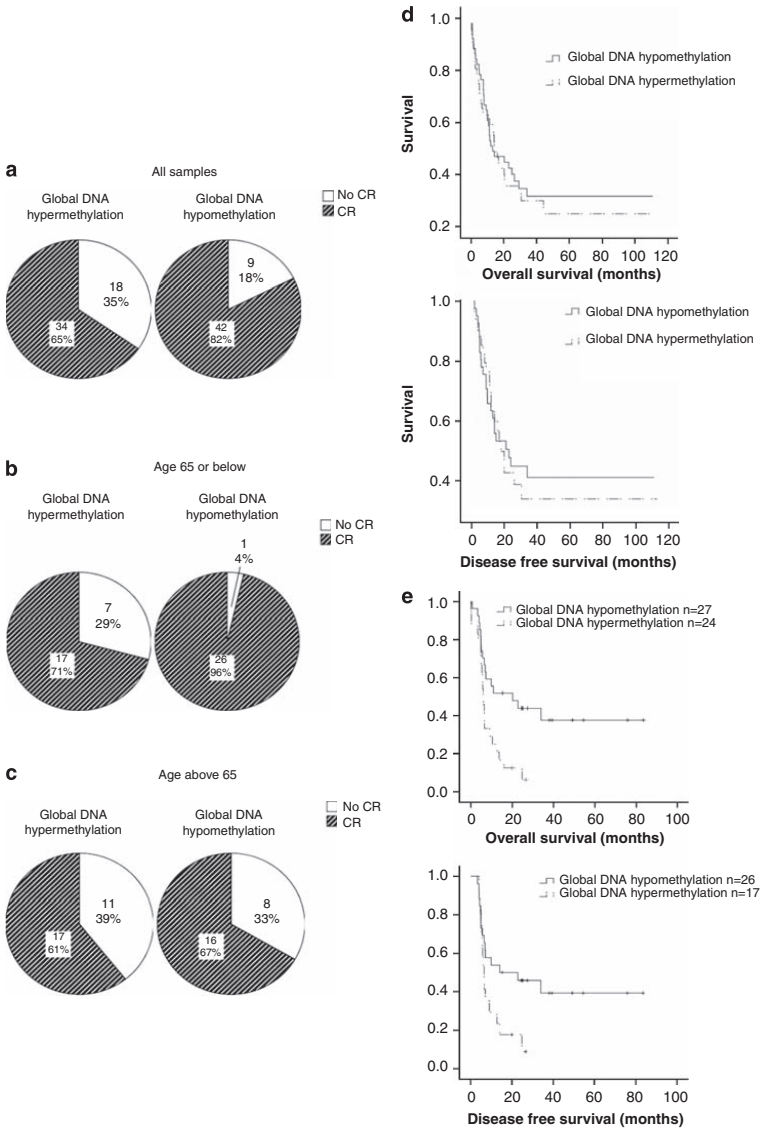

Figure 8 CR rates are higher in patients whose samples show global hypomethylation. The pie charts show CR rates in samples with global methylation levels higher than median ('global DNA hypermethylation') and global methylation levels lower than median ('global DNA hypomethylation'). (a) The whole AML cohort (P=0.05), (b) the same data for AML patients younger than 65 years (P=0.02) and (c) for AML patients older than 65 years (P=0.7). (d) Overall survival (P=0.9) and disease-free survival (P=0.6) in the whole AML cohort according to high or low global DNA methylation by LUMA. (e) In patients younger than 65 years, OS (P=0.005) and DFS (P=0.04) were significantly better in samples with low global LUMA methylation levels. Patients were censored at the time of allogeneic stem cell transplantation.

4.3 METHYLATION OF CDKN2B MEASURED BY DGGE CORRELATES WITH IMPROVED SURVIVAL IN AML

In paper I, DNA methylation analysis by DGGE showed methylation of the CDKN2B (p15) promoter in 66%, of CDH in 66% and HIC1 in 51% of the evaluable AML cases. Only p15 and HIC1 methylation correlated with each other (R=0.33, P=0.001) and only p15 methylation associated with overall and disease free survival, but not CR rate (figure 9).

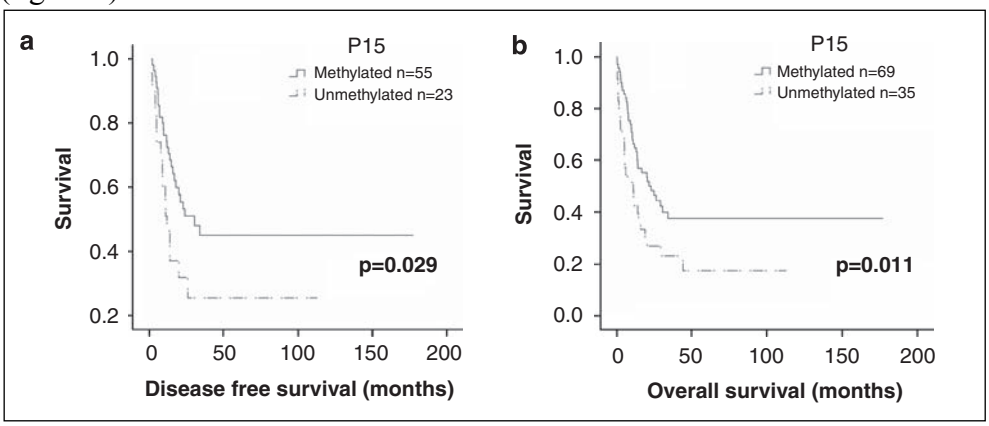

Figure 9 Kaplan–Meier diagrams showing the prognostic effect of CDKN2B (p15) promoter methylation by DGGE on (a) disease-free survival (n=104) and (b) overall survival (n=78)

The DGGE analysis was validated by bisulfite sequencing of the p15 gene promoter, which was concordant in 23/29 samples evaluated with both methods (79%, P=0.003 Chi^2) and with MS-MCA in nine samples, which were 100% concordant. Previous studies evaluating the relationship between methylation of the p15 promoter and survival in AML show conflicting results. Methylation of the p15 promoter has been associated with poor prognosis[160, 268, 269] whereas some studies have failed to show any association with prognosis[270, 271]. Other studies support our findings, associating more methylation with better prognosis in AML[272]. This concept has been shown previously in colon cancer, in which the CpG island methylated phenotype subtype is associated with improved survival[273]. We believe that the diverging results between our findings and the studies mentioned above are due to differences in the selection of patients and methylation analysis techniques. Recent data, including our own observations, have illustrated the differences between MDS, MDS–AML and de novo AML regarding methylation patterns [166, 215, 274], and we suggest them to be analysed separately. There is no information of the frequency of MDS related or secondary AML cases in the study by Shimamoto et al, and in the study by Wong et al, the adverse risk of p15 promoter methylation in the adult AML subset was actually not significant (P=0.145)[269]. The recent study by Alvarez et al. utilized Illumina Golden Gate array technology validated with methylation specific-PCR (MS-PCR) found that p15 promoter methylation was an adverse prognostic factor, however not retained in multivariable analysis[160]. We believe that the use of MS-PCR, as for instance by Shimamoto et al.[268], is problematic. DGGE is less sensitive than methylation-specific PCR but most likely better reflects the general methylation levels in the highly variable promoter region[275]. In addition, methylation-specific PCR may pick up very low levels of methylation that are not physiologically relevant. Even so, we believe that

further studies of the relevance of p15 promoter methylation on prognosis in AML are needed before the matter is resolved.

4.4 GENOME WIDE METHYLATION PATTERNS MEASURED WITH ILLUMINA ARRAYS ASSOCIATE WITH SPECIFIC MOLECULAR MUTATIONS IN CN-AML

In paper II, 58 cases of CN-AML were investigated with Illumina HumanMethylation27k arrays. To find differentially methylated CpG sites, we applied a Bayes modified, Benjamini-Hochberg adjusted t-test for difference set to less than 0.05 and a minimum geometric average distance between groups of β >0.10. With these criteria, 2764 CpG residues corresponding to 2304 genes were found to be differentially methylated. Unsupervised hierarchical clustering of the samples based on these was performed showing six sample clusters with unequal distribution of NPM1 and IDH mutations (P=0.01 and P=0.0001, respectively, Fisher-Freeman-Halton test). Results are shown graphically in figure 10.

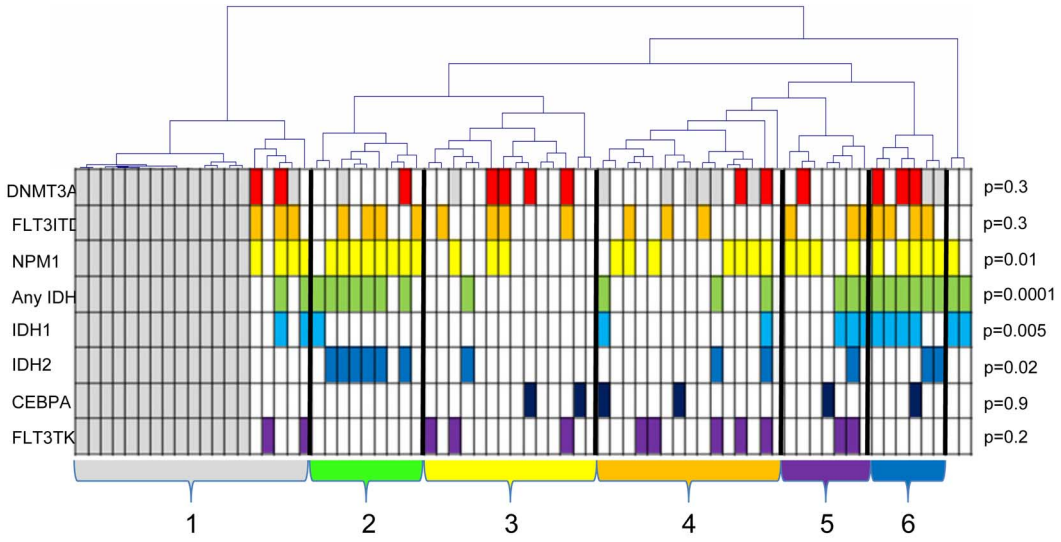

Figure 10 Unsupervised complete linkage hierarchical clustering of samples according to methylation of the 2764 differentially methylated CpG sites. There are 2 outliers and 6 major clusters of samples. The normal samples clustered together within cluster one (grey) and are marked by grey boxes below the dendrogram. Mutational status is indicated below; white boxes represent wild-type; and colored boxes, mutations. P values are given for unequal distribution between clusters for each mutation using the Fisher-Freeman-Halton test (outliers were disregarded). The frequency of NPM1 mutations is increased in cluster 2 and 6, IDH1 in cluster 6, and IDH2 in cluster 2.

The clustering according to different mutations is not surprising and has been described in another well performed study[148]. Interestingly, IDH1 and IDH2 mutations clustered in different groups, and in univariate analysis both were associated with increased average β-levels (p=0.006), but in IDH1 mutated samples, only CpG-island annotated sites showed increased methylation (P=0.02), whereas in IDH2 mutated samples, only non-CpG island sites were hypermethylated (P=0.0002). Furthermore, Polycomb Group (PcG) associated targets were preferably methylated in IDH1 mutated compared with IDH2 mutated samples. The mechanistic link behind this is not clear. However, IDH1 is a cytosolic enzyme and IDH2 mitochondrial[276], making it

feasible that their influence on DNA methylation would differ. This is a novel finding that is quite interesting, not least since IDH1 and IDH2 mutations are almost mutually exclusive[131, 132].

4.5 GENOME WIDE METHYLATION PATTERNS MEASURED WITH ILLUMINA ARRAYS ASSOCIATE WITH CLINICAL OUTCOMES IN CN-AML

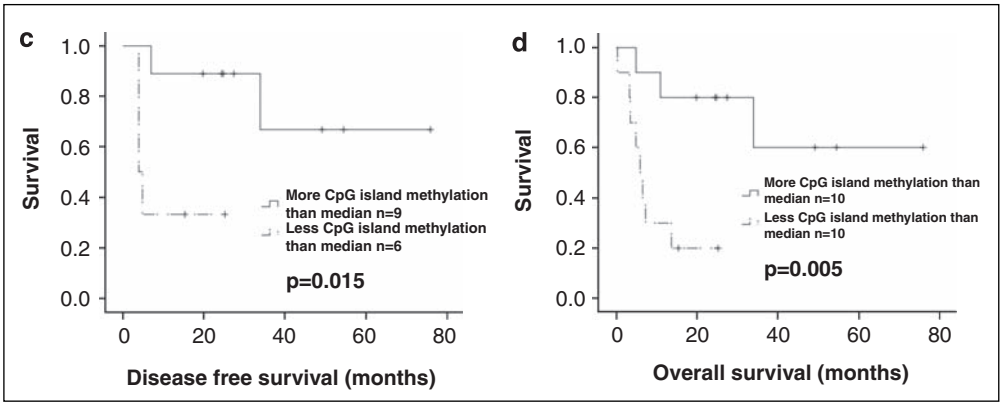

Figure 11 Prognostic value of the number of CpG sites hypermethylated according to the Illumina Methylation array, divided at median, for (c) disease-free survival (n=15) and (d) overall survival (n=20).

Most significant for CR	Most significant for 2 year survival
AIFL	ADORA3
ARMC3 *	AGPAT2
BIK *	AMT
Bles03	ANGPTL2
Bles03	ANK1
C6orf125	AVP
CARD15	BHMT *
CCDC38	BM88
CD302	C10orf72
ChGn *	C12orf34
CTSW	C14orf29
DEFB118	C1orf172
DGKA	C20orf98
DHH	C20orf98
EMR2	C5orf4
FCGR1A *	CACNA2D2
FLJ33860	CHST13 *
FLJ45909 *	COL21A1 *
GPR128 *	CSF3
GPR92 *	CST11 *
GSTP1	DHCR24
IL27	DKK4
INHBC	DYNC1I1
INT1	ESM1 *
KIAA0020	ETNK2 *
KRTAP19-1	FAM57A
LAT2 *	FBLIM1
LCP1 *	FGF1
LXN	GAL3ST3 *
ME2	GALNT5
MGC15875	GDPD5
MMP7	GNMT
NKG7 *	GNMT
NLGN2	GPR17
OXGR1 *	HSD3B7
PLD4	KCNA1 *
RCSD1	KCNA6 *
TCP11	KCNK4 *
TIGD2	MEGF10 *
TRAF1 *	MGC39715 *
UNQ9356	MGC39715 *
ZNF134	NAB1
	NEUROG1 *
	NIP
	OR2B6
	OXGR1 *
	PDGFRB
	PLA2G3
	PPP1R9A
	PRP2
	PYGM
	RRAS2
	SLC44A4
	SLC4A11 *
	SNCAIP *
	STAR
	SYDE1
	TCF15 *
	TF *
	TOM1L1 *
	ZNF354C
	ZNF513

Table 5 The most significantly differentially methylated genes for achievement of complete remission (CR) and 2 year survival. * marks PcG target genes.

In paper I a rough analysis of the average β-values of the 20 samples that were subjected to Illumina array analysis proved to significantly correlate with overall and disease free survival. The finding was unexpected and serendipitous, but nevertheless significant (figure 11).

The patients selected for Illumina array investigation were all under 67 years of age, meaning that roughly the same patients that were divided by LUMA global methylation values were similarly divided in this analysis, given the correlation between the analyses (see section 4.2 and figure 8). In paper II, in the test cohort of 58 patients we could not repeat the prognostic implication of average β-values of the Illumina 27k arrays. However, when refining the analysis and limiting it to the PcG target genes, we found that the average β-values of these was a strong and independent prognostic factor and, importantly, we could validate this in an independent cohort of 60 patients, figure 12. It may well be that in paper I, the average β-values of the whole Illumina 27k array, and also the LUMA values, were a proxy for the PcG target gene methylation status. One plausible

explanation of the powerful prognostic impact could be the central role of Homeobox (Hox) gene methylation in AML pathogenesis. Hox genes encode DNA binding proteins central in embryonic development and hematopoiesis, and their expression is epigenetically regulated by PcG and Trithorax proteins[277]. They are highly enriched in our cohort among the differentially methylated genes. Hox genes are overexpressed in AML with mixed lineage leukemia translocations, which is a marker of poor prognosis[278]. Several authors have found that high expression of Hox genes correlates with poor prognosis and low expression with favorable prognosis, compatible with our results[279, 280]. Other possible explanations are offered when looking at the most significant genes associated with outcome (Table 5), many of which are PcG targets. Interestingly, several potassium channel encoding, PcG-targeted, genes were on the list of methylated genes associated with 2-year survival. Potassium channels have been associated with AML prognosis, with low expression being a favorable prognostic factor[281, 282].

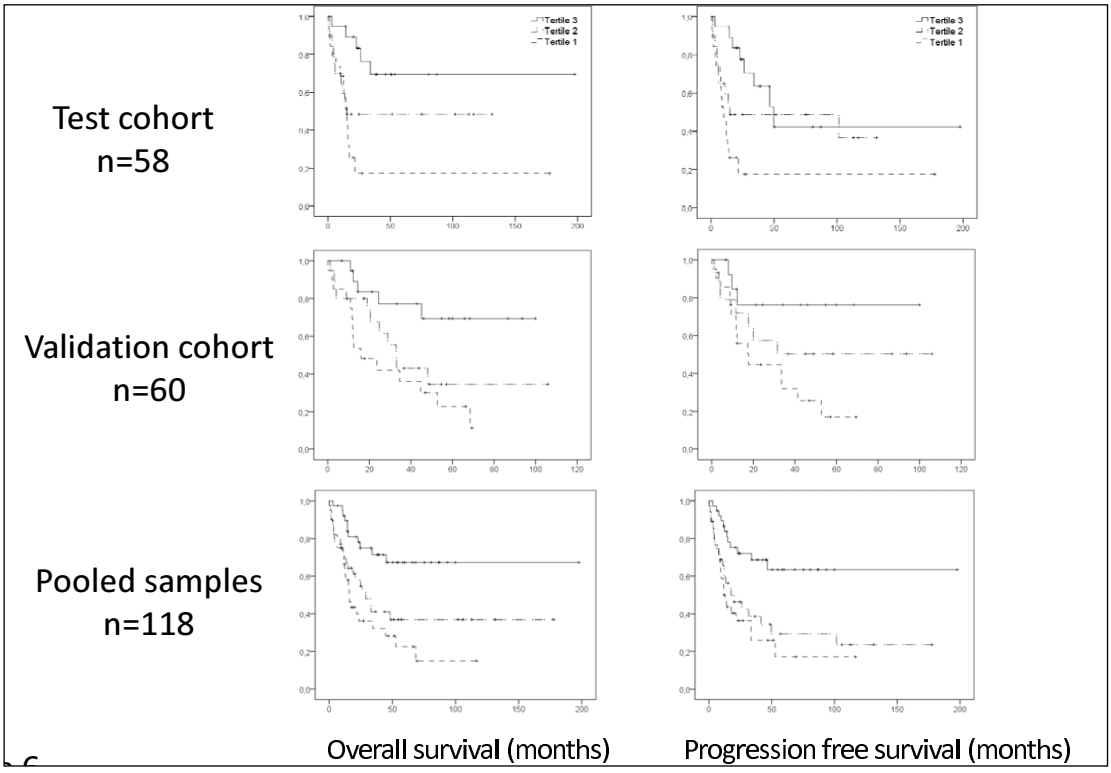

Figure 12 Kaplan-Meier diagram showing the impact of the methylation levels of PcG-marked genes on OS and PFS. The samples were divided in tertiles according to the average methylation levels of PcG-marked genes. The tertile with most methylation (tertile 3), marked with a solid line, had significantly better OS (C) and PFS (D) than the less methylated tertiles; in the test cohort (top panels): P(trend)=0.001 and 0.002, respectively. In the validation cohort (bottom panels): P(trend)=0.009 and P(trend)=0.035 (middle panels), and for pooled samples: P(trend)=0.00009 and 0.0002, respectively.

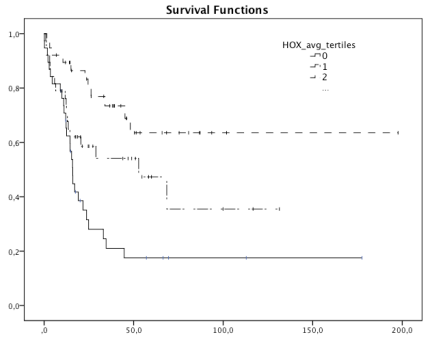

Figure 13 Kaplan-Meier diagram showing the impact of the methylation levels of HOX genes on OS. The samples were divided in tertiles (38 in each) according to the average methylation levels of PcG-marked genes. The tertile with most methylation (tertile 3), marked with a dashed line, had significantly better OS. P(trend)=0.00003

A further analysis of the implication of clustered (cluster A-D) Homeobox (HOX) gene average methylation levels revealed that they were an as strong predictor of overall survival as all PcG targeted genes (figure 13).

4.6 ABERRANT METHYLATION OF THE BTG4 / miR 34-b/c PROMOTER IN CLL WITH FUNCTIONAL CONSEQUENCES

In a previous study in CLL, SNP-chip technique demonstrated a large commonly deleted region at 11q, similar to what has been reported previously[283]. However, one case with 11q-deletions showed two small deleted regions with a small heterozygote region in between. One of the deleted regions contained the ATM gene whereas the other contained 6 other genes (POU2AF1, BTG4, FLJ46266, LAYN, SNFLK2 and PPP2R1) as well as microRNA-34b/c (miR-34b/c). BTG4 and miR-34b/c are directed from a shared two-way promoter that has been characterized in detail by Toyota et al in colon cancer[284]. In CLL with deletion of the 11q region, the ATM gene is implicated as a tumor suppressor, but is only mutated in 30% of CLL cases. There is circumstantial evidence that there is another tumor suppressor gene in the region and several have been suggested[226, 285, 286]. We searched the genes of the microdeletion for epigenetic silencing and found a high degree of aberrant methylation in the BTG4 – miR34b/c promoter, 25/52 (48%) with MS-MCA. None of the other genes has similar degrees of aberrant methylation (table 6). To validate the MS-MCA findings, primers for bisulfite pyrosequencing were constructed and 29 of the investigated samples including two healthy controls were sequenced. The primer locations are shown graphically in relation to BTG4 and miR-34b/c in figure 14. Results were highly concurrent with the MS-MCA results (p=0.001, Mann-Whitney).

Gene	Methylation screening	Methylation Extended screen
POU2AF1	0/10	-
BTG4-miR34b/c	4/10	25/52
FLJ46266	N/A – no CpG island	-
LAYN	2/10	2/15
SNF1LK2	0/10	-
PPP2R1	0/10	-

Table 6 Aberrant methylation screening in CLL. Number of positive / negative cases evaluated with MS-MCA.

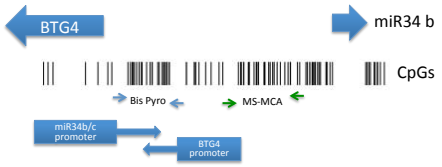

Figure 14 CpG sites (vertical bars) and primers in relation to the BTG4 and miR-34b/c gene and promoter regions.

Interestingly, there was no significant correlation with 11q deletions; 19/35 (54%) were methylated of non-11q deleted compared to 5/16 (31%) of 11q deleted CLL cases (p=0.126). There was a trend towards increased methylation in IgVH mutated samples, 63% vs. 39% (p=0.099). No correlations between del17p, trisomy 12, del13q or the

combination of 11q and 17p deletions and BTG4/miR-34b/c methylation were found. Importantly, when we stress incubated primary CLL cells with Doxorubicin, we found that miR-34b/c, but not BTG4, was up-regulated in samples that were unmethylated compared to methylated samples, P=0.038 (figure 15, upper panel). This effect was not found, perhaps attenuated, when the TP53 activating agent PRIMA1 or the demethylating agent Decitabine was used for incubation, P=0.6 (figure 15, middle and lower panel). Furthermore, cases with miR-34b/c methylation had a better overall survival than non-methylated cases (figure 16). The significance of methylation however disappeared when a multivariable Cox regression model including IgVH-status, age and 11q status was applied. The miR-34b/c analogue miR-34a is located on chromosome 1p and has been implicated as a tumor suppressor in CLL[287], making the discovery of methylation of the miR-34b/c promoter highly interesting. miR-34a and the clustered miR-34b/c are transcriptional targets of p53 and reintroduction of miR-34s in deficient cells increases apoptosis and decreases growth[288]. Furthermore, miR-
34b/c are silenced by hypermethylation in oral squamous cell carcinoma, gastric cancer, malignant melanoma and colon cancer[284, 289-291] and are considered tumor suppressors in these cancers[292]. The shared promoter of BTG4 and miR-34b/c makes it difficult to determine which of these genes have tumor suppressive functions only by methylation analysis. We therefore conducted functional experiments, showing that a TP53-activating drug, doxorubicin, selectively induced miR-34b/c transcripts but not BTG4, implying a functional role in drug resistance or apoptotic mechanisms, previously shown for miR-34a[287]. Somewhat surprising, we did not find any associations between miR-34b/c methylation and 11q or 17p deletions. This may be due to low power as only 6 samples with 17p deletion were included in the study. Still, a trend could be seen towards more methylation in IgVH mutated samples (p=0.099). The finding is somewhat provoking since ATM mutations and also p53 mutations occur almost exclusively in IgVH unmutated CLL, leaving the door open for other oncogenic mechanisms in IgVH mutated cases, where miR34b/c methylation is more common. This would also be compatible with our observation of better survival in BTG4/miR-34b/c mutated patients, a prognostic factor that became non-significant when corrected for age, deletion 11q and IgVH status.

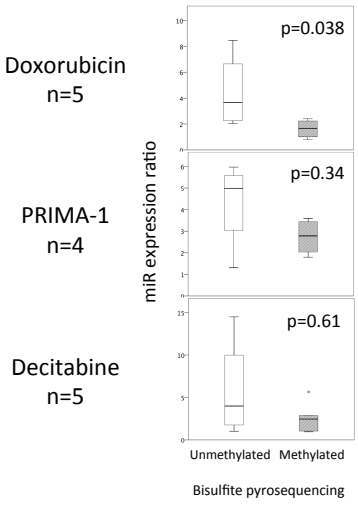

Figure 15 Panels show miR-34b/c expression measured as the expression ratio (normalized to RNU6) of drug vs. mock incubated samples. Panels on the left show miR-34b/c levels in relation to methylation status.

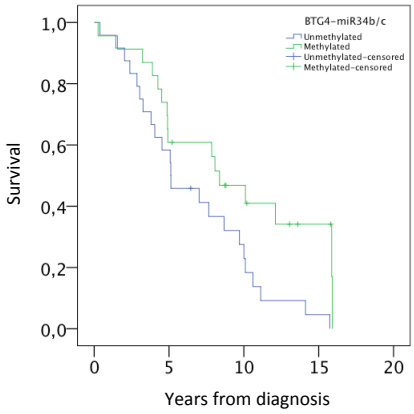

Figure 16 Kaplan-Meier curve of overall survival of 47 patients with methylated (green) of unmethylated (blue) BTG4/miR-34b/c promoter. Methylation at the BTG4-miR34b/c locus was associated with significantly improved overall survival (P=0.024, Log-Rank)

5 CONCLUSIONS

Paper I: Our results show that low global DNA methylation levels measured with LUMA correlate with an improved CR rate and that methylation of p15, measured with DGGE, may be an independent prognostic factor in AML. We also show an inverse correlation between global DNA methylation and genome-wide promoter methylation and an association between the number of hypermethylated CpG sites measured genome-wide by the Illumina HumanMethylation27 array and prognosis.

Paper II: In conclusion, we show that NPM1 and IDH mutations associate with specific clusters of samples, that PcG target genes have an increase of aberrant methylation compared with other genes, and that the level of PcG target methylation may be an independent prognostic factor for clinical outcome in CN-AML. We suggest that methylation patterns may be used as new tools to predict outcome in AML patients and that there are potentially new subgroups of AML that could be defined by methylation patterns.

Paper III: We show for the first time that miR-34b/c promoter, located at the commonly deleted region of chromosome 11q, is aberrantly hypermethylated in nearly half of CLL cases, regardless of karyotype, but with a trend of less methylation in IgVH unmutated cases. We also show that silenced miR-34b/c expression can be induced by stress signals such as a through exposure to a cytotoxic drug, an effect that seem to be dependent on methylation status. However, further studies on the importance of miR-34b/c methylation in CLL are needed.

6 FUTURE PERSPECTIVES

6.1 IS THERE AN EPIGENETICALLY DRIVEN SUBSET OF AML? A HYPOTHESIS

Our paper I and paper II show that epigenetic changes in AML have prognostic implications, and in paper II the most important epigenetic changes for prognosis can be narrowed to the Polycomb group targeted genes. Further analysis, presented in this thesis (figure 13), implies that the clustered Homeobox (HOX) genes are the core set of these prognostic PcG target genes. These are genes that are important in embryogenesis but also pivotal for hematopoetic and myeloid differentiation. They are expressed in an orderly and strictly regulated fashion, meaning that epigenetic impairment of one may lead to silencing or disorderly repression of other HOX genes and non coding-RNAs "caudally" in the same cluster, even if these genes in themselves were not functionally incapacitated. Given the results of our research with better survival of CN-AML cases with HOX gene methylation, we can formulate a testable hypothesis; that *epigenetic impairment of HOX genes is a common mechanism effectuating differentiation block in AML*.

Larger studies of DNA methylation of HOX and PcG targeted genes in AML samples well characterized cytogenetically and molecularly could further define the context of this epigenetic mechanism and, importantly, define its prognostic role in relation to allogeneic stem cell transplantation, opening the possibility for clinical utilisation in therapeutic decision making. Further functional studies and the development of a cell line system that could be utilized for drug screening, would open up for translation of this new knowledge into clinically useful applications.

6.2 ESTABLISHING THE ROLE OF MICRO-RNA-34B/C IN CLL

Our paper III deals with the role of miR-34b/c as a plausible tumor suppressor gene on chromosome 11q in CLL. To conclude whether this is a true tumor suppressor further functional experiments should be undertaken, both knock out experiments and rescue knock in, preferably using transfection of anti-sense RNA and micro-RNAs in a cell line model, observing the impact on proliferation and apoptotic ability. If proven to be a tumor suppressor gene, the possibilities of utilising synthetic oligomers, for instance locked nucleic acids (LNA:s), as therapeutic agents is a fascinating road ahead.

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