Epigenetic regulation of oncogenes and tumor suppressors in chronic lymphocytic leukemia

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Declare the past, diagnose the present, and foretell the future

-Hippocrates

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ABSTRACT

DNA methylation is one of the well-known epigenetic modifications. Aberrant DNA methylation has been shown to have a major role in tumorigenesis and is associated with tumor aggressiveness and inferior outcome in various cancer types. Chronic lymphocytic leukemia (CLL) is the most common adult leukemia characterized by the accumulation and clonal expansion of long-lived neoplastic B-lymphocytes. It is clinically and biologically a very heterogeneous disease. The specific aim of study I is to investigate the role of the tumor suppressor gene, Microcephalin (*MCPH1*) in regulating the expression of the Angiopoietin gene (*ANGPT2*) in CLL. We showed that MCPH1 negatively regulates *ANGPT2* gene, which is overlapping with *MCPH1* in opposite direction through a novel mechanism. MCPH1 physically binds to the *ANGPT2* promoter and recruits the DNA methylation machinery for subsequent silencing of *ANGPT2*. Study II is mainly focused on epigenetic silencing of *miR26A*1 microRNA and its impact on Enhancer of zeste homolog 2 (EZH2) in CLL and mantle cell lymphoma (MCL). We showed that *miR26A1* acts as a tumor suppressor and epigenetically silenced in CLL, which is required for maintaining high levels of EZH2, resulting in poor overall survival. Finally, in study III we analyzed the mechanisms behind Ten-eleven-translocation 1 (*TET1*) deregulation in CLL. Here we characterized mechanisms that control *TET1* gene activity at the transcriptional level. Overall, we proposed a model by which the *TET1* gene activation in CLL depends on miR26A1 regulated EZH2 binding at the *TET1* promoter and silencing of a novel cryptic promoter through gene-body

hypermethylation. In conclusion, these three studies deepen our knowledge in understanding the functional role of DNA methylation controlled tumor-related genes in CLL, resulting in the identification of potential prognostic biomarkers and target for therapy.

Keywords: DNA methylation, epigenetic modifications, CLL, MCPH1, ANGPT2, EZH2, miR26A1, TET1, MCL, tumor suppressor, gene-body, expression, prognostic biomarkers

SAMMANFATTNING PÅ SVENSKA

Metylering är en av de mest välkända epigenetiska modifieringarna av DNA. Avvikelser i metyleringsmönstret har visats ha en viktig roll vid malign transformation och är associerad med försämrad prognos vid flera olika cancersjukdomar. Kronisk lymfatisk leukemi (KLL) är den vanligaste leukemiformen hos vuxna och karaktäriseras av ansamling och klonal expansion av neoplastiska B lymfocyter. Sjukdomen är både kliniskt och biologiskt heterogen. I artikel 1 var syftet att undersöka vilken roll tumörsuppressorgenen Microcephalin (*MCPH1*) har i transkriptionell reglering av genen Angiopoietin (*ANGPT2*) vid KLL. Vi visar att MCPH1 hämmar transkription av *ANGPT2*, som sker överlappande med *MCPH1* fast i motsatt riktning. Detta sker genom att MCPH1 fysiskt binder till promotorn till *ANGPT2* och därefter rekryterar metyltransferas som deltar i DNA-metylering av promotorn, vilket hämmar transkription av *ANGPT2*. Artikel 2 har sitt fokus på epigenetisk nedreglering (epigenetic silencing) av miR26A1 och dess effekt på Enhancer of zeste homolog 2 (EZH2) vid KLL och mantelcellslymfom (MCL). Vi visar att miR26A1fungerar som en tumörsuppressor, då epigenetisk nedreglering av miR26A1 leder till förhöjda nivåer av EZH2 och försämrad prognos vid KLL. Slutligen utreder vi i artikel 3 mekanismerna bakom dysreglering av Ten-eleven-translocation 1 (*TET1*) vid KLL. Här karaktäriseras mekanismer som är inblandade i transkription av genen *TET1*. Vi lägger fram en modell i vilken aktivering av *TET1* vid KLL beror av miR26A1 reglerad inbindning av EZH2 till promotorn till *TET1*. Denna inbindning hämmar expression, genom hypermetylering av genens introniska regioner, en tidigare okänd kryptisk promotor för ett transkript i motsatt riktning. Sammanfattningsvis har dessa studier bidragit till fördjupad förståelse för betydelsen av DNA-metylering av gener inblandade i utveckling av KLL, vilka har potential att användas som prognostiska biomarkörer eller för riktad behandling.

LIST OF PAPERS

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

I. **Kopparapu PK**, Miranda C, Fogelstrand L, Mishra K, Andersson PO, Kanduri C, Kanduri M. MCPH1 maintains long-term epigenetic silencing of ANGPT2 in chronic lymphocytic leukemia. FEBS J. 2015; 282 :1939-52

II. **Kopparapu PK**, Bhoi S, Mansouri L, Arabanian LS, Plevova K, Pospisilova S, Wasik AM, Croci GA, Sander B, Paulli M, Rosenquist R, Kanduri M. Epigenetic silencing of miR-26A1 in chronic lymphocytic leukemia and mantle cell lymphoma: Impact on EZH2 expression. Epigenetics. 2016; 11: 335-43.

III. **Kopparapu PK**, Morsy MHA, Kanduri C, Kanduri M. Gene-body hypermethylation controlled cryptic promoter and miR26A1-dependent EZH2 regulation of TET1 gene activity in chronic lymphocytic leukemia. Oncotorget. 2017; 8: 77595-608

Related paper that is not included in this thesis

Wernig-Zorc S*, **Kopparapu PK***, Bemark M, Kristjansdottir H, Andersson PO, Kanduri C and Kanduri M. Global distribution of DNA hydroxymethylation and DNA methylation in chronic lymphocytic leukemia (Under review) *Shared equal contribution

CONTENT

ABBREVIATIONS

AID	Activation-Induced Deaminase
ANGPT2	Angiopoietin 2
ATP	Adenosine TriPhosphate
Bcl-2	B-Cell Lymphoma 2
caC	Carboxyl Cytosine
CDRs	Complementarity-Determining Regions
CLL	Chronic lymphocytic leukemia
CpG	Cytosine phosphate Guanine
DNMT	DNA Methyltransferase
EZH2	Enhancer of Zeste Homolog 2
fC	Formyl Cytosine
hmC	Hydroxylmethylcytosine
hmU	Hydroxylmethyluracil
Ig	Immunoglobulin
IGHV	Immunoglobulin Heavy-chain Variable
LINEs	Long Interspersed Transposable Elements
mC	Methylcytosine
MCPH1	Microcephalin
MCL	Mantel Cell Lymphoma
miR	MicroRNA

MYOD1	Myoblast Determination Protein 1
ncRNA	Non-coding RNA
NK	Natural Killer
PRC	Polycomb Repressive Complex
RAG	Recombinase Activating Gene
RB1	Retinoblastoma 1
RSS	Recombination Signal Sequence
SINEs	Short Interspersed Transposable Elements
SMAD1	Mothers Against Decapentaplegic Homolog 1
TdT	Terminal deoxynucleotidyl Transferase
TET1	Ten-Eleven Translocation-1
TGF	Transforming Growth Factor
Tp53	Tumor Protein p53
ZEB	Zinc Finger E-box-Binding Homeobox

1 INTRODUCTION

1.1 Epigenetics and its modifications

The human body consists of more than 200 types of cells. Each cell type maintains a unique cellular identity represented by the particular transcriptional program. There must be strict regulation of gene expression in each cell within the human body as the genetic material is essentially identical in almost all the cell types in the body. Transcriptional regulation is carried out by controlling the accessibility to genes which is achieved by the packaging of DNA into particular arrangements. The DNA contains the genetic information encoded by the sequential order of four nucleotides: adenine (A), guanine (G), thymidine (T) and cytosine (C). DNA is wrapped around proteins called histones, which are structurally organized and condensed into chromatin. Chromatin is a complex dynamic structure of DNA, proteins (histones) and RNA. Histone proteins can be modified and these modifications serve as important regulators in the transcription of necessary genes throughout the life cycle of an organism.

‘’Epigenetics’’ term was coined by Conrad Hal Waddington in 1942 and defined as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being’’[1] and recently it is defined as “the mitotically and /or meiotically stable heritable changes in gene expression that are caused by mechanisms other than alterations in the genetic sequence”[2]. Waddington explained the cellular levels of differentiation arising from common progenitors culminating in a fully formed organism. Epigenetic changes refer to “somatically heritable modifications to the genome that do not involve changes in the nucleotide sequence that leads to altered gene expression’’. In fact, all cells of a complex multicellular organism contain the same genetic information, but during development, each single cell differentiates into a specific phenotype and remembers the genetic information without any changes in DNA sequence. This feature of epigenetics implies that the accuracy of epigenetic modifications is vital for maintaining the genome integrity and the cell phenotype. Abnormal regulation in epigenetic mechanisms tends to induce various complex diseases including cancer, neurodegenerative disorders, obesity, cardiovascular diseases etc., Indeed, epigenetics contributes to the

understanding of mechanisms underlying different diseases for which genetic mutations are not the only cause. Epigenetic modifications are essential for normal development and the maintenance of gene expression patterns in mammalian cells. Failure to maintain heritable epigenetic marks results in inappropriate activation or inhibition of signaling pathways, leading to certain specific disorders. Epigenetic modifications are dependent on changes in chromatin structure which defines how genetic information is organized within a cell[3]. DNA methylation, histone modifications, nucleosome remodeling, and non-coding regulatory RNAs (including microRNAs) are the examples of such modifications[4]. Interaction and co-operation between these different modifications regulate the accessibility and compaction of chromatin, resulting in modulating gene expression. This thesis mainly addresses the epigenetic pattern at the level of DNA methylation in chronic lymphocytic leukemia (CLL) along with the implication of chromatin modifier which is Enhancer of zeste homologue 2 (EZH2) in addition to non-coding RNAs and how such epigenetic pattern in CLL regulates oncogenes and tumor suppressors.

1.1.1 DNA methylation

Methylation of DNA occurs at the 5'-carbon of a cytosine (5mC), commonly when preceding guanine (CpG) (Figure 1). In mammals, cytosine methylation is primarily restricted to the symmetrical CpG context. DNA methylation mainly targets CpGs that can be clustered in CpG rich regions (CpG islands), be scattered in regions with less condensed CpGs around the CpG islands (CpG shores) or be found as individual CpGs. Methylation of the fifth position of cytosine is the best studied and mechanistically understood epigenetic modifications and is well conserved among most plant, fungal and animal models. Three evolutionary conserved enzymes, DNA methyltransferase 1 (DNMT1), DNMT3A and DNMT3B are responsible for its deposition, maintenance and are essential for normal development[5,6]. The interconnection between DNA methylation and other epigenetic modifications such as histone modifications results in strong functional effects on regulating gene expression. DNA methylation marks are established during embryonic development and are copied to the following generation through DNA replication. Interestingly, due to the environmental factors such as nutrition, exercise, metabolites, exercise, chemical agents can change these patterns over time[7]. DNA methylation plays a crucial role in

regulating several important factors in genome stability, X-chromosome inactivation, suppression of retrotransposon elements[8], mammalian development[6] and the regulation of gene expression in a specific cell during the different phases of the cell cycle[9]. Since DNA methylation is reversible, DNA methylation inhibitor drugs such as 5-azacytidine (5-azaC) and 5-aza-2'-deoxycytidine (5-azadC), were tested as anticancer drugs with the idea that such agents would demethylate and reactivate tumor suppressor genes. However, these agents might cause activation of a group of prometastatic genes in addition to activating tumor suppressor genes, which might lead to increased metastasis[10].

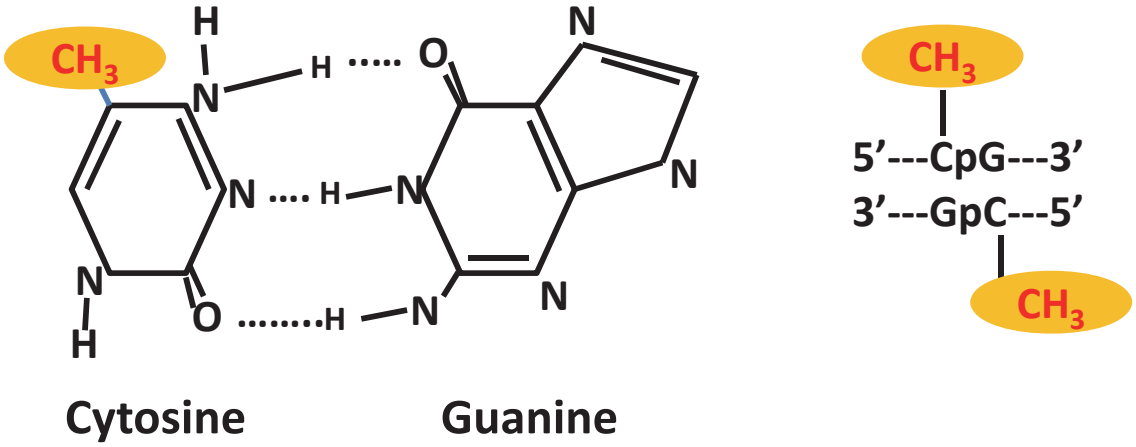

Figure 1: *Model showing the inclusion of methyl group (CH3) on 5' carbon of cytosine in C_pG dinucleotide context. Modified figure from* [11]

DNA demethylation

The first discovery of methylcytosine (5mC)[12] was thought to be a stable epigenetic mark until the discovery of hydroxymethylation (5hmC) in mouse and human brains[13]. DNA methylation is a stable epigenetic mark which can only be reversed by inhibiting the maintenance enzyme during cell divisions (passive DNA demethylation), DNA methylation can be reversed in an active manner through the consecutive oxidation reactions (active DNA demethylation). With the help of DNMTs, cytosine is converted into methylcytosine. DNMT3A/B are the most important regulators for *de novo* methylation in early development processes, whereas DNMT1 maintains DNA methylation patterns through successive rounds of cell division[14]. Once established, the global DNA methylation patterns must be stably maintained

in order to ensure that transposons remain in a silenced state and to preserve cell-type identity. DNMT1 is associated with replication foci and functions to restore hemimethylated DNA generated during DNA replication to the fully methylated state[15].

Oxidation of DNA has traditionally been considered a DNA damage event, which is readily removed by DNA repair pathways. It has been well demonstrated that enzymatic oxidation of 5mC to 5hmC by TET proteins may act as a stable modification of DNA and downstream removal of 5hmC may actually be part of a complex and intricate process of epigenetic gene regulation. TET proteins are 2-oxoglutarate (2OG) - and Fe (II)-dependent enzymes that catalyzes 5mC into 5hmC, 5formylcytosine (5fC), 5carboxylcytosine (5caC) by three consecutive oxidative reactions[16].

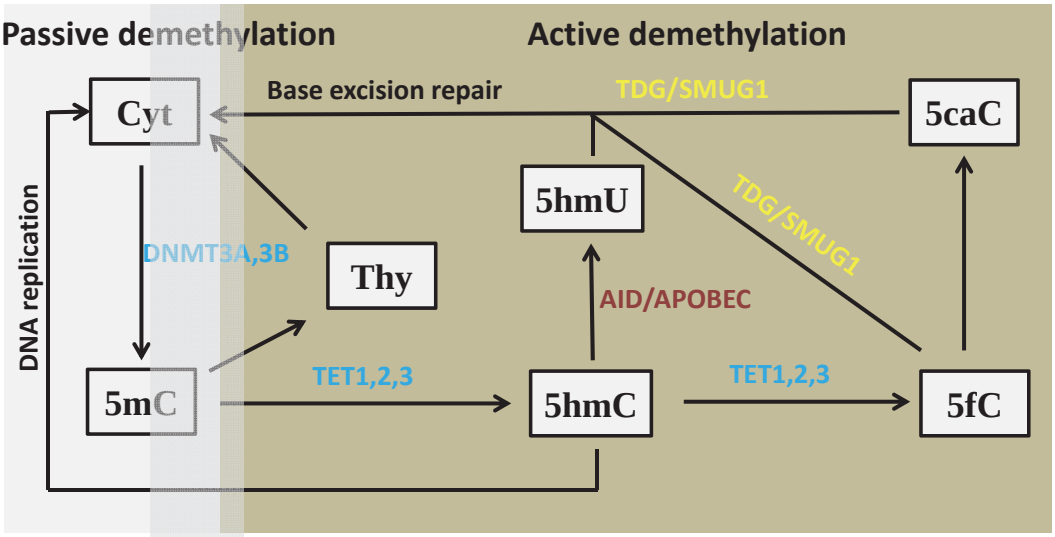

Figure 2: *DNA demethylation dynamics. Modified figure from*[17-19]

Further 5fC and 5caC are recognized by TDG proteins which activate the base excision repair pathway. There might be other proposed mechanisms for active DNA demethylation by enzymes like activation-induced deaminase (AID) which deaminates 5hmC, or Gadd45a to 5hmU, but this is still subject of debate[20] (Figure 2).

DNA methylation and Cancer

Proper establishment and maintenance of DNA methylation patterns are essential for both embryonic developments and for the normal functioning of the adult organism. DNA methylation is a potent mechanism for silencing

gene expression and maintaining genome stability in the face of a vast quantity of repetitive DNA, which can otherwise mediate irregular recombination events and cause transcriptional deregulation of nearby genes causing various disorders including cancer, imprinting disorders, fragile X syndrome, immunodeficiency, centromeric instability and facial anomalies syndrome[3], Alzheimer's disease[21] and cardiovascular diseases[22].

One of the hallmarks of cancer is DNA methylation deregulation associated with global changes in gene expression levels. In a normal cell, DNA methylation occurs predominantly in repetitive genomic regions, including satellite DNA and parasitic elements (such as long interspersed transposable elements (LINEs), short interspersed transposable elements (SINEs) and endogenous retroviruses)[23] and the methylation at individual CpG sites (mCpG) is established in a regional-specific manner, with some regions that remain unmethylated and others that are hypermethylated. In a cancer cell, this established methylation pattern is drastically modified, with some DNA regions undergoing methylation resulting in hypomethylated areas and some DNA regions undergoing *de novo* methylation resulting in hypermethylated areas. In a cancer cell, hypomethylation is linked with DNA instability and gene activation, whereas gene silencing and gene mutation are linked with hypermethylation in a cancer cell[11] (Figure 3). Along with abnormal DNA methylation patterns in cancer, recent studies has highlighted on mutations that encounter genes the products of which are involved in epigenetic regulators such as DNA methyltransferases, DNA demethylases, histone methyl- and acetyltransferases and in histone H3. Cytosines within CpG islands, especially those associated with promoter regions are less methylated in normal cells and lack of methylation in promoter-associated CpG islands allows gene transcription to occur, facilitating the binding of appropriate transcription factors maintaining open chromatin structure[24]. The first-observed epigenetic change in human cancer was a loss of DNA methylation throughout the genome were compared with a wide variety of normal tissues[25]. Seemingly, cancer cells are associated with global hypomethylation but with regional hypermethylation of CpG islands at gene promoters[26]. Abnormal genome-wide hypomethylation may relate to tumorigenesis by promoting genomic instability. DNA methylation of promoter CpG islands is associated with a closed chromatin structure and transcriptional silencing of the associated gene[24,27]. Loss of methylation may contribute to tumorigenesis in several ways including loss of imprinting[28], generation of chromosomal

instability[29] and reactivation of normally methylated oncogenes[30]. In chronic lymphocytic leukemia (CLL), based on genome-wide DNA methylation studies, hypomethylation occurred more frequently in gene body including introns, exons, and 3'-UTRs[31].

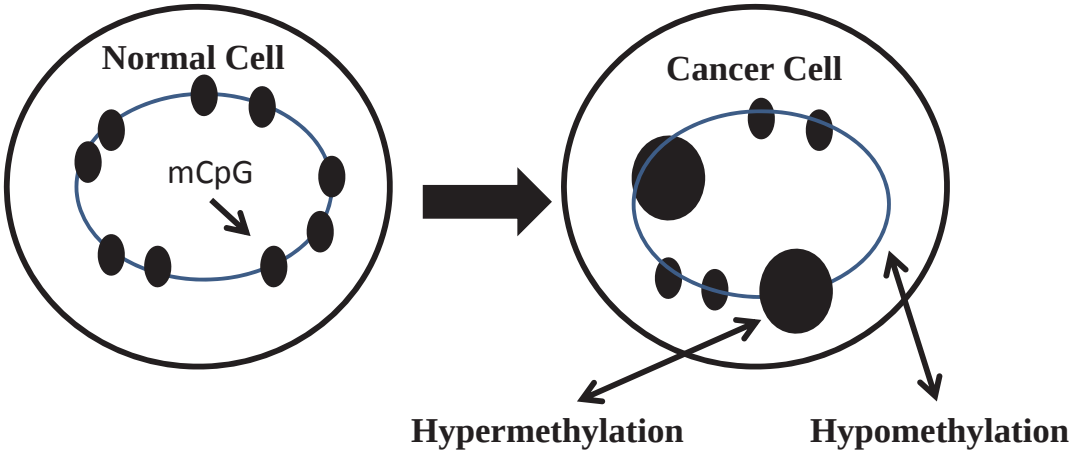

Figure 3: *Model showing global DNA methylation status in normal and a cancer cell. Modified Figure from* [11]

1.1.2 Histone modifications

The size of DNA in a human diploid cell is approximately 2 meters (6 feet) if stretched. Interestingly, this long DNA is compacted and condensed in the nucleus of each cell with the help of histone proteins. Histones are small proteins with a positive charge, the main types of histones involved in compacting DNA are H1, H2A, H2B, H3, and H4; however, there are histone variants as well which have their own functions. Given that histones carrying net positive charges, DNA whose backbone is negatively charge is wrapped nearly twice around histones.

Histones have protruding N-terminal tails which post-translationally can undergo chemical modifications known as histone modifications/marks. Histone modifications (depending on the residue they occur on, the type of modifications, and the number of modifications) are associated with the active or repressed state of genes[32]. Indeed, certain histone modifications can be used to predict gene expression[33]. Several histone modifications are described, including acetylation, methylation, ubiquitination, phosphorylation and sumoylation[34].

1.1.3 Nucleosome remodeling and chromatin modifiers

The nucleosome is the fundamental unit of chromatin composed of 150bp of DNA wrapped into histone proteins[35]. Chromatin presents a significant barrier to the interaction of trans-acting factors with DNA in majority of cases where chromatin regulates many biological processes such as DNA replication, transcription, DNA repair, and DNA recombination. Histone modifications are added and removed by a group of enzymes that work in coordination with chromatin remodelers[36]. Nucleosome remodeling and the incorporation of histone variants are mediated by the action of ATP-dependent chromatin remodeling complexes[37,38]. Chromatin remodeling complexes major function is to control gene expression by allowing access of condensed genomic DNA to the regulatory transcription machinery proteins[39,40]

The Polycomb Group of proteins (PcG) form complexes that modify the chromatin, maintaining gene repression during development and differentiation. Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2) are part of the polycomb group of protein complex. Enhancer of zeste homologue 2 (EZH2) is one of the core members of the PRC2 complex involved in catalyzing silencing histone mark, which is H3K27me3. For certain genes, EZH2 mediated repression and DNA methylation are coordinated in order to maintain gene silencing. However, EZH2 can also directly control DNA methylation by recruiting DNA methyltransferases onto the target genes[41], moreover, genes marked by PRC2-EZH2 are major targets for DNA methyltransferases. Many studies suggest that EZH2 acts as an oncogene and is aberrantly overexpressed in several types of cancer including in several hematological malignancies[42-44].

1.1.4 Non-coding RNAs mediated regulation

The other epigenetic modifiers include non-coding RNA mediated gene silencing (ncRNAs). Misregulations of these RNAs are involved in many biological processes and have gained increasing importance in the past few years. ncRNAs are usually classified based on their mature length, location and orientation according to the nearest protein-coding gene, and their function which could be either cis or trans. The functional non-coding RNAs may include ribosomal RNA, transfer RNA, microRNAs (miRNAs, ~19-24

nucleotides), or long-noncoding RNAs (lncRNAs, > 200 nucleotides)[45]. Since my work is mainly involved in microRNA gene regulation; I would elaborate more on this particular class of small non-coding RNAs.

MicroRNAs

The first miRNA family *lin-4* was identified in C. elegans through a genetic screen for defects in the temporal control of post-embryonic development[46]. MicroRNAs (miRNAs) are class of small (~19-24 nucleotides) non-coding RNAs which are highly conserved among mammals that guide post-transcriptional repression of mRNA targets[47,48]. miRNAs play crucial roles in cell differentiation, proliferation, apoptosis, tumorigenesis and host-pathogen interactions[49-51].

In humans, miRNA sequences are located within various genomic contexts: the majority of canonical miRNAs are encoded by introns of coding or noncoding transcripts, but some miRNAs are encoded by exonic regions. Often, several miRNA loci are in close proximity to each other, constituting a polycistronic transcription unit[52]. The miRNAs in the same cluster are generally co-transcribed, but the individual miRNAs can be additionally regulated at the posttranscriptional level. Nearly the same fraction are hosted within and co-transcribed as part of a protein-coding messenger RNA (mRNA) transcription unit (TU). Classically, in the nucleus, these primary miRNA (pri-miRNA) are initially clipped out of the nascent TU by the microprocessor complex [DROSHA (RNase III enzyme) and DGCR8]. DGCR8 is believed to bind to a single-stranded portion of the pri-miRNA located at the base of the double-stranded stem, opposite the loop. Guided by DGCR8, DROSHA cleaves the pri-miRNA ~11 nucleotides (nt) into the stem, releasing a precursor miRNA (pre-miRNA) hairpin product comprised of the stem and loop[53]. Primary miRNA and RNA splicing processing appears to be tightly coordinated in the case of intron-resident miRNAs, with evidence pointing to miRNA “cropping” proceeding intron removal. The resulted pre-miRNA intermediate is shuttled out of the nucleus through the Exportin-5 nuclear transport receptor in cooperation with a Ran GTPase cofactor[54]. In the cytoplasm, the pre-miRNA is cleaved by Dicer, another RNase III-like enzyme, which generates mature miRNAs that ranges from ~19-25nt with 2-nucleotide 3′ overhangs on both ends (Figure 4).Various transcription factors such as p53, MYC, ZEB1 and ZEB2 and myoblast

determination protein 1 (MYOD1) as well as epigenetic regulators positively or negatively regulate miRNA expression[55].

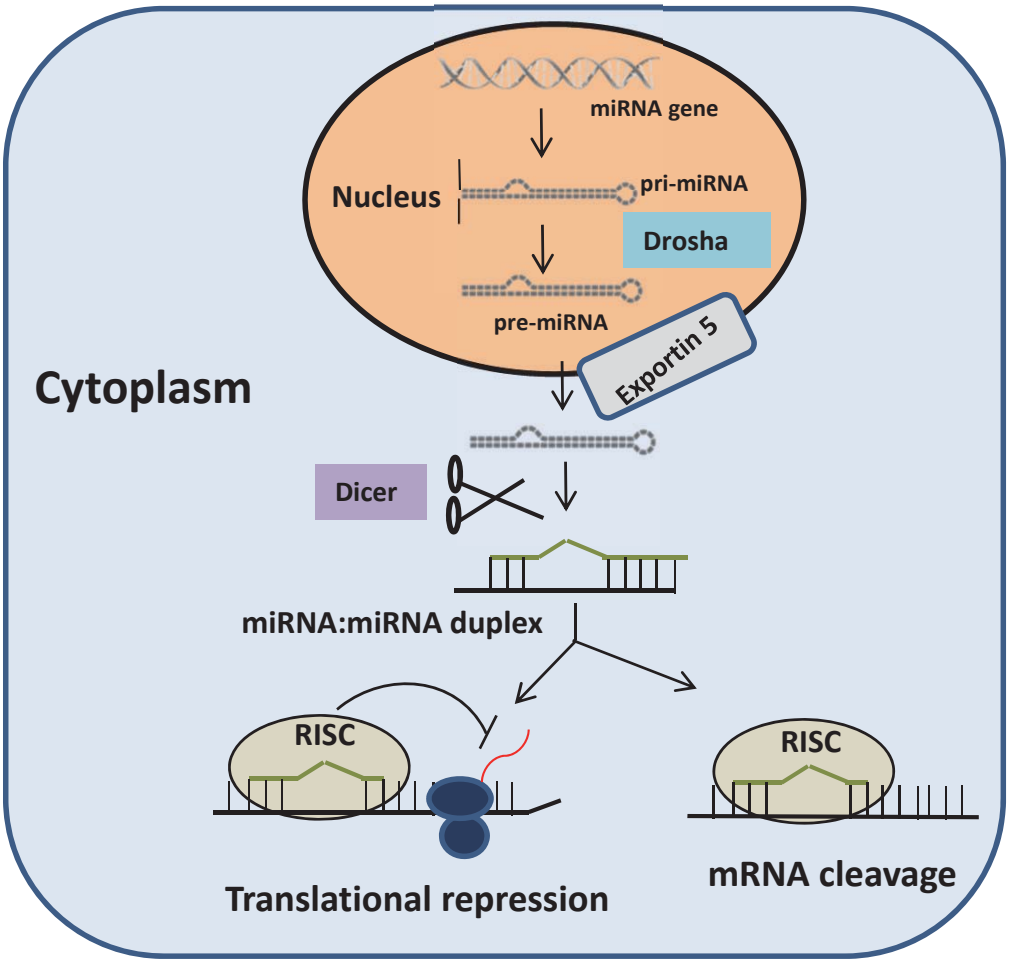

Figure 4: *Model showing the biogenesis and post-transcriptional suppression of microRNAs. Modified figure from*[56,57]

Alteration of microRNA expression profiles occurs in most cancers, as approximately half of human microRNAs are located at fragile sites and genomic regions involved in alterations in cancers, suggesting that individual microRNAs could function as tumor suppressors[58] or oncogenes[59]. Many recent studies have shown frequent deregulation of miRNAs occurs in various human malignancies including various leukemia[59-66], lymphomas[47,67,68]. miR26A1 is a known tumor suppressor microRNA shown to be involved in pathways such as p53 and TGF beta pathways regulating several transformation-related targets, like SMAD1[69] and EZH2[70].

1.2 B-cell Malignancies

Mature B-cell malignancies represent heterogeneous group of diseases with distinct genetic, phenotypical and clinical features. It is known that various lymphomas and leukemia occurs when the regulation of B-cell differentiation and activation is altered. These include follicular lymphoma, Burkitt lymphoma, multiple myeloma, diffuse large B-cell lymphoma, marginal zone lymphoma, mantle cell lymphoma and chronic lymphocytic leukemia[71,72]. This thesis is mainly focused on chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), both belonging to B-cell malignancies, but in many ways represents extremes within the spectrum of B-cell lymphomas. More detailed information on these two malignancies are described below.

In order to know the classification and pathogenesis of CLL and MCL, sufficient knowledge on developmental stages of their counterparts is warranted. B-cells and T-cells arise from the hematopoietic (lymphoid) stem cell which is crucial to the human body's ability to protect against infection and cancer by producing antibodies that attack pathogens and removing infected cells. B-cells play a pivotal role in clearing and preventing infection as well as offering protection against antigens[73]. Autoimmunity, malignancy, immunodeficiencies and allergy commonly occurs due to defects in B-cell development, selection and function[74].

In mammals, B lymphopoiesis takes place in the bone marrow (BM) from hematopoietic precursor cells, which depends on the interaction of stromal cells and growth factors, where they undergo VDJ rearrangement of the variable region of their immunoglobulin genes and start to express surface immunoglobulin (sIg)[75]. V-D-J rearrangement takes place at the first B-cell subset called the pro-B-cell. At this stage, CD19 is expressed and the IG heavy chain (IGH) chain locus starts to rearrange. Following this the pro-B-cell becomes a pre B-cell and expresses the μ heavy chain on its surface. The pre–B-cells undergo 1 or 2 cell divisions and rearrange the gene segments encoding the k and λ chains. An IgM molecule is formed and expressed on the cell surface when combined with the μ chain and these cells are termed immature B-cells[76]. The immature B-cells leave the BM and migrate to the periphery blood as so-called mature B-cells. Mature B cells circulate in the periphery for few days or weeks and die if they do not encounter an antigen. These mature B-cells are called naïve B-cells co-express IgD and IgM and

start to circulate and recognize foreign antigens (Figure 5). This naïve B cell enters the lymphoid organs after the encounter with antigen and is activated in the outer T cell zone. Then the B cell can either becomes a plasma cell, secreting antibodies or enters primary follicles where it participates in giving rise to germinal centers (GCs)[76,77]. There are two zones in the GC called the dark and light zone in which the B cell differentiation and selection takes place[78].

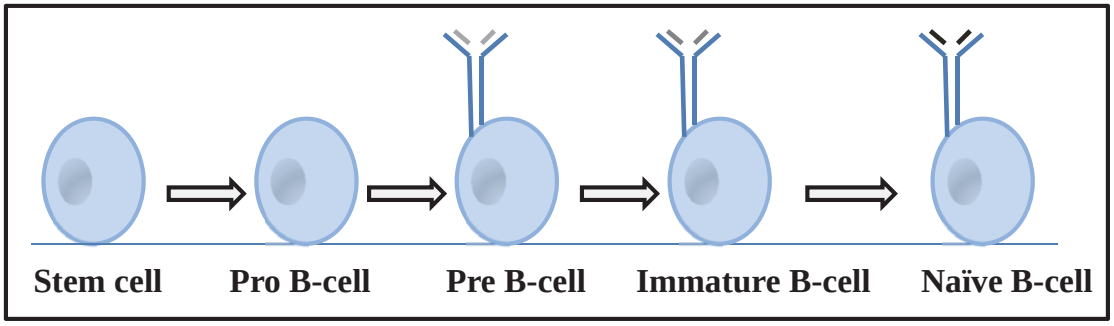

Figure 5: *Different stages of B-cell development*

During B-cell development, rearrangement of genetic segments of IGH gene loci which encodes the heavy chain of immunoglobulin takes place. The term immunoglobulin was proposed by Herman in 1959 for those globulins primarily associated with the lymphoreticular system[79]. Immunoglobulin consists of two heavy chains (IGH) and two light chains (IGL) which are linked together by disulfide bridges near the carboxy-terminal grouping of the light chain[80] (Figure 6). Each component chain contains one NH2-terminal variable (V) domain and one or more COOH-terminal constant domain (C). The three distinct gene segments which encode the heavy chain of the variable region includes variable (VH), diversity (D) and the joining region (JH) genes, whereas two segments-variable (V_K or V_λ) and joining (J_K or J_λ) region genes-encode the light chains[81,82]. The IGH gene locus is located on chromosome 14q32.33 and the two IGL loci namely k and λ are located on chromosome 2 and 22, respectively[83,84]. Each V gene segment typically contains its own promoter, a leader exon, an intron, an exon that encodes the four framework regions (FRs 1, 2, 3 and 4), complementarity-determining

regions (CDRs) (CDR1, 2 and 3) and a recombination signal sequence (RSS). Each joining (J) gene segment begins with its own recombination signal, the carboxy-terminal portion of CDR3, and the complete. The initial event during IGH gene rearrangement juxtaposes a D region segment to a JH segment. After successful IGH- DJ recombination, a VH region gene rearranges to the D–JH complex as IGHV. The heavy chain C region remains separated from the rearranged IGHV-DJ complex by an intron, and this entire sequence is transcribed. After the productive rearrangement of at least one heavy chain gene, transcription of the rearranged locus occurs. μ heavy chain protein is expressed on the surface of pre-B-cells after translation[85]. These total gene recombination processes depend on the activity of enzymes. The creation of a V domain is directed by the RSSs that flank the rearranging gene segments which are dependent on the protein products of the recombinase activating genes (RAG-l and RAG-2)[86-88]. Both RAG1 and RAG2 introduce a DNA double-strand break between the terminus of the rearranging gene segment and its adjacent RSS and then these breaks are repaired by DNA repair process known as non-homologous end joining (NHEJ). Terminal deoxynucleotidyl transferase (TdT) one of the nuclear enzymes can variably add non–germline encoded nucleotides to the coding ends of the recombination product providing junctional diversity[89].

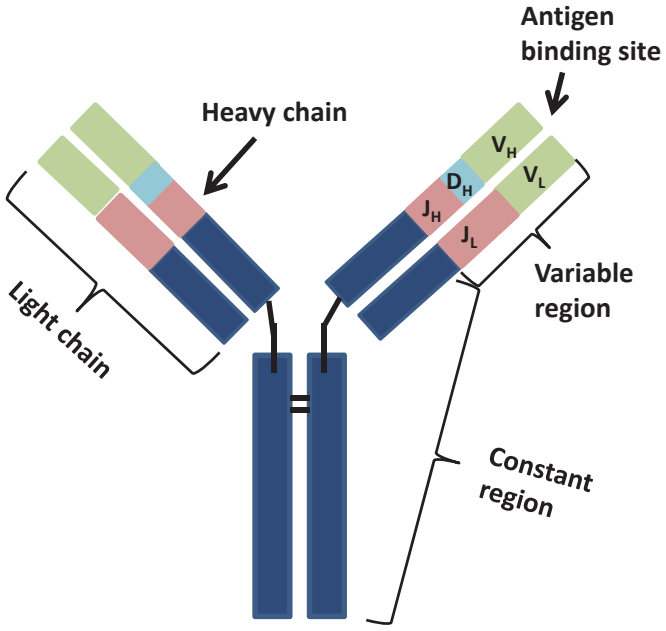

Figure 6: *Structure of immunoglobulin. Modified figure from*[90-92]

If one of the heavy chains in IGH rearrangements is unsuccessful during B-cell development, recombination will initiate at the second one and if productive, these cells will then mature into pre-B-cells. If this rearrangement also fails, cells will undergo apoptosis[85,93].

1.2.1 Chronic lymphocytic leukemia

CLL is the most common adult leukemia in western countries with approx. 550 new patients diagnosed annually in Sweden[94] and it is estimated that CLL will account for 20,110 new cases and 4,660 deaths in the US alone in 2017[95].

CLL is characterized by the accumulation of mature B lymphocytes specifically by the clonal expansion of CD5+ CD23+ in blood, bone marrow and secondary lymphoid tissues[96,97]. CLL is most frequent in elderly people over the age of 60 with a median age 65 years and the incidence rate in men are twice as high as in women[98-100]. The incidence rates with people of African-Caribbean descent and Asian-Pacific show lower incidence than American-Europe descents. The reason behind this is still elusive but may reflect a combination of genetic and environmental factors[101]. Most CLL patients are often asymptomatic, commonly diagnosed in routine or other medical check-up and symptoms in advance disease include fatigue, fever, enlargement of lymph node, anemia, leukopenia, weight loss, night sweats and bone marrow failure[100]. The preliminary diagnosis is done in the presence of $\geq$ 5000 B-cells/microliter of peripheral blood for more than 3months[102] followed by immunophenotyping: the composite immunophenotype CD5+, CD19+, CD20+ (low), CD23+, sIg low, CD79b low, FMC7– allows the distinction of most cases of B-cell type CLL from other CD5+ B-cell lymphoma[103-105].

1.2.1.1 CLL cell of origin

CLL is classified into two prognostic groups based on the presence or absence of somatic hypermutations in the *IGHV* genes namely IGHV mutated (indolent and good survival rate) and IGHV unmutated (aggressive and poor prognosis)[106,107]. Based on the observation that CLL cells carry either IGHV mutated or unmutated, it is proposed that M-CLL and UM-CLL cells are derived from two distinct populations: IGHV M-CLL cells are derived from the antigen-experienced post-germinal center B-cells, in the presence of T cell help. In contrast, IGHV UM-CLL is pre-GC derived from marginal zone

B-cells by T-cell independent processes[106]. However, evidence from gene expression profiling on CLL cells found that both IGHV mutated and unmutated cells share a common gene-expression profile suggesting they have a common origin and have a similar phenotype bearing markers of memory B-cells[108,109]. While there is no clear information the phenotype of the B-cells that clonally expands to generate CLL, evidence from recent studies suggests that the earliest genetic and epigenetic alterations leading to CLL may actually occur in pluripotent hematopoietic stem cells[110] (Figure 7). *In vivo* experiments show that in mice injected with purified patient CLL-HSCs but not CLL-pro or mature CLL can engraft efficiently into immunodeficient mice and cause the generation of CD19+ CD5+ CLL like B-cell clones with IGHV-DJ combinations.

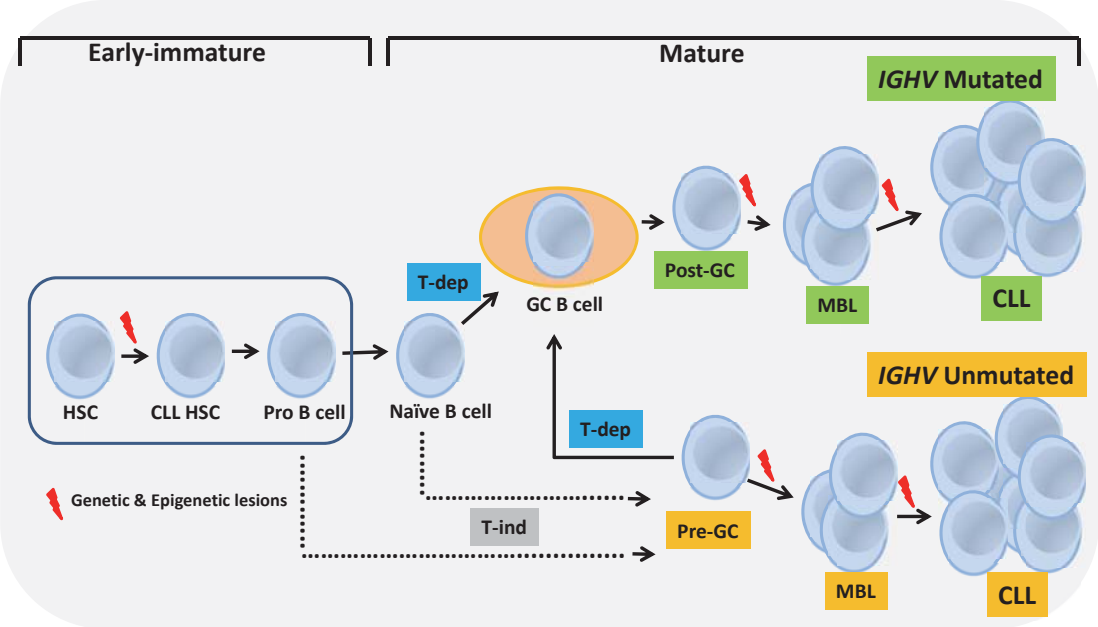

Figure 7: *The cellular origin of CLL. Modified figure from*[97,99]

Along with these, CLL-associated genetic lesions have been found in multipotent progenitors from patients with CLL, stating that the cellular origin of CLL occurs in a stepwise pattern that is initiated at a much earlier stage. Aberrations in HSC with predispositions for B-cell ontogenesis are suggested leading to polyclonal expansion at the pro-B-cell stage, and then to oligoclonal CD5+, monoclonal B-cell lymphocytosis (MBL), with each step leading to the acquisition of new malignant properties. Based on Kikushige *et al's* model, progression from MBL to CLL requires further oncogenic events. IGHV mutated CLL seem to originate from post-GC CD5+ CD27+ B-cells

that have undergone GC reaction, whereas IGHV unmutated CLL seem to originate from pre-GC CD5+CD27- B-cells which may derive from the separate lineage of precursor B-cells or naïve B-cells[97,110,111].

1.2.1.2 Prognostic markers

According to recent studies, the median survival of CLL patient was about 6 years or more, although individual survival varies based on various factors[112,113]. Currently, there has been enormous progress in the identification and characterization of molecular and cellular markers that may predict the tendency of disease progression or detect minimal residual disease after therapy in CLL patients.

Clinical staging

So far the two widely used clinical staging systems which are recommended by the international workshop[102] on CLL are Rai (0-IV)[114] and Binet (A-C)[115]. Patients with Rai 0/ Binet A normally survive more than 10 years, Rai I/II/Binet B survive 5-7 years while Rai III/IV/Binet C survive ≤ 2years. These staging systems are based on the clinical characteristics of patients with CLL[116,117]. However, these staging systems fail to predict the higher risk of progression among patients in early stages of the disease and also identifying the prognostic subgroups and response to therapy[118].

Chromosomal aberrations

All patients with CLL do not have a single cytogenetic defect. Today with the use of fluorescence *in situ* hybridization (FISH) more than 80% of patients with CLL show one or more cytogenetic aberrations. The most common chromosomal aberrations include deletion of 13q14, trisomy 12, deletion of 11q22–23 and deletion of 17p13 (Table 1). In 2000, Dohner *et al* showed the survival analysis with various chromosomal aberrations based on FISH analyses of 325 CLL patients. A total of five different categories were defined: 17p deletion, 11q deletion, 12q trisomy, normal karyotype and 13q deletion as the single abnormality where the median overall survival was 32, 79, 114, 111 and 133 months respectively and median treatment-free survival ranges between 9 and 92 months[119] (Figure 8). According to multivariate

analysis, deletions of 11q22-q23 and 17p13, resulting in abnormalities of *ATM* (ataxia-telangiectasia mutated) and *TP53* (encodes a tumor suppressor protein p53) genes, respectively, are independent prognostic factors identifying patients with a rapid disease progression with a poor overall survival. Deletion 11q patients tend to have an advanced clinical stage and lymphadenopathy. Deletion of 13q14 is associated with a good prognosis while trisomy 12 has an intermediate overall survival. Moreover, the deletions of 11q and 17p are frequently detected among the patients with unmutated *IGHV* genes at an advance stage of disease[120-122]. Interestingly, even though *TP53* is located on 17p, there is no direct relation between them. In a study, patients having deletion 17p did not found *TP53* while some patients having *p53* mutation do not have a 17p deletion. In both cases, the CLL outcome showed poorer survival similar to carrying both 17 deletion and p53 mutation, suggesting the importance of p53 act as independent prognostic marker[123]. Patients with deletion 13q14 with higher frequency have a good prognosis, with survival curves that are even better than those with a normal karyotype. Two micro-RNA genes, namely *miR15* and *miR16* at 13q14, are absent or down-regulated in most cases of CLL. *miR15* and *16* have been shown to target BCL-2 as part of the normal control of gene expression, and their absence in CLL appears to be a major factor in preventing apoptosis[122,124,125]. These major cytogenetic abnormalities have been used as prognostic categories for the most relevant risk estimation in patients with CLL.

Table 1: *The major chromosomal aberrations in CLL*

Chromosome	Genes involved	Frequency (%)	Category
Del 13q14.3	*miR15, miR16,*	>50	Low risk
Del 11q22-q23	*ATM*	19	High risk
Trisomy 12	*MDM-2*	15	Intermediate risk
Del 17p13.3	*p53*	15	Very high risk

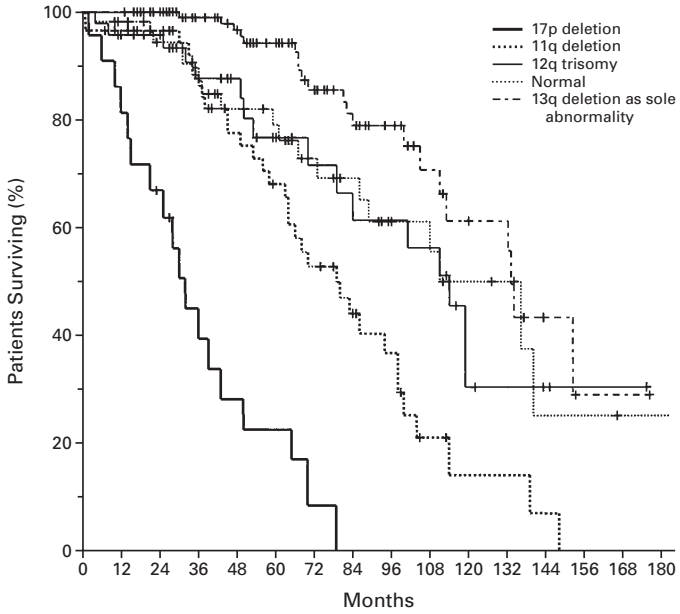

Figure 8: *Overall Survival from the Date of Diagnosis among the Patients based on five Chromosomal aberrations. Reproduced with permission from*[119]*, Copyright Massachusetts Medical Society.*

IGHV gene mutational status

In 1999, two independent groups, namely Damle *et al* and Hamblin *et al,* reported that CLL patients can be divided into two prognostic subtypes based on the degree of somatic hypermutation and percentage of homology with *IGHV* gene sequence[107,126]. Patients with greater than 98% homology with *IGHV* gene sequence were designated as IGHV unmutated (with a median survival of 8 years from diagnosis), while patients with 98% or less homology with *IGHV* gene sequence were designated as IGHV mutated (with a median survival of 25 years). Patients with *IGHV* unmutated genes tend to have a more aggressive malignant condition, including adverse cytogenetic features (i.e., deletion 11q, deletion 17p), clonal evolution, resistance to therapy and poorer survival than those with mutated *IGHV* genes (Figure 9). IGHV mutations are independent with cytogenetic aberrations and clinical stages regarding prognostic significance particularly in patients with early-stage disease[127]. However, exceptions do exist and that is the expression of the *IGHV3-21* gene, which is associated with a poorer outcome independently of the IGHV mutational status[128]. Today, although many investigators believe that the mutation status of the *IGHV* genes is the best predictor of outcome, on a practical level, sequencing the *IGHV* is labor-

intensive, time-consuming and expensive which led to research efforts to identify surrogates for the mutational status, specifically CD38 and ZAP-70.

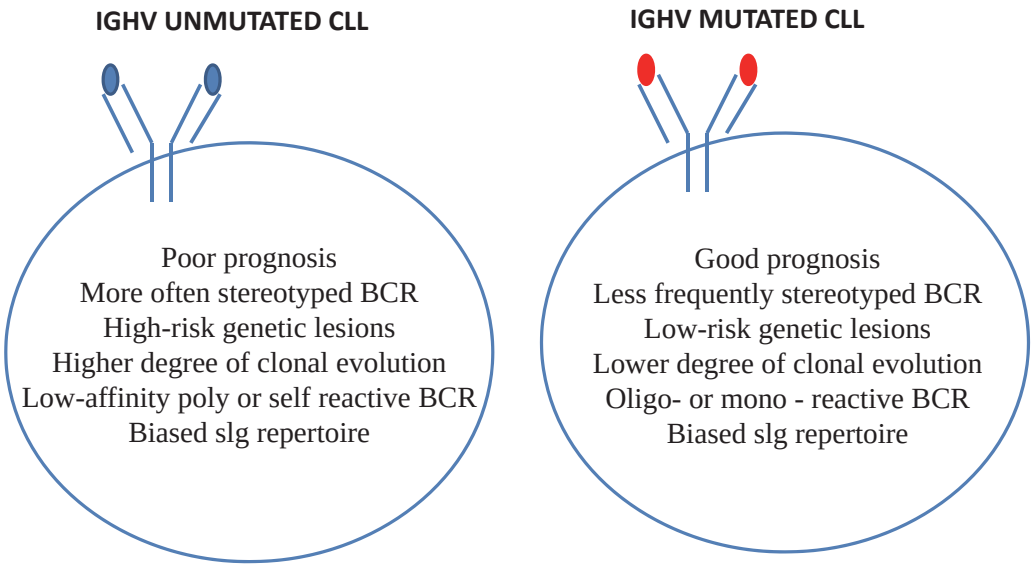

Figure 9: *Comparison between IGHV mutated and unmutated prognostic subgroups. Modified from* [99]

Prognostic markers based on gene expression

CD38

CD38 is transmembrane glycoprotein which is 45kDa in humans which expresses at the cell membrane in high levels by B lineage progenitors in BM, B lymphocytes in the germinal center, activated tonsils, and by terminally differentiated plasma cells[129,130]. CD38 is also found in different areas of the brain, pancreatic acinar cells, smooth muscle cells, osteoclasts, although in most of these instances, CD38 is located in the cytosol and/or in the nucleus but not on the cell membrane[130]. CD38 plays a vital role in cell adhesion, calcium flux into the cell, proliferation and also influences B-cell apoptosis through BCR signaling[131-133]. Low levels of CD38 express in mature and memory B-cells. Damle *et al*, was the first group in CLL to show that expression of CD38 correlates with an aggressive mutation status[107] and this was confirmed by subsequent studies[134,135]. CD38 expression as a prognostic factor on its own has also been shown to have unfavorable clinical outcomes with a decreased response to chemotherapy, shorter time to initiation of first treatment and decreased survival[135]. CD38 acts as an independent prognostic marker but is not the strongest marker due to its

limitation regarding the appropriate threshold to define CD38 positivity (5%, 7%, 20% and 30%)[118,136] and its expression change over the disease course[137,138]. Lately, various studies have shown CD49d, a protein that mediates cell-cell interaction in CLL also co-expressed on CLL cells proving that prognostic value of this marker[139,140].

ZAP-70

Zeta-chain-associated protein kinase 70 (ZAP-70), a 70-kDa T-cell receptor-chain associated protein tyrosine kinase is the best surrogate marker for IGHV mutation status. ZAP-70 higher expression is observed in normal NK and T cells, while the expression on normal B-cells is low or absent. In a study, Rosenwald *et al*. found that a small number of genes allow separating mutated and unmutated CLL, encoding most of the genes with ZAP-70. The majority of IGHV mutated cases are ZAP-70 negative, while unmutated cases are ZAP-70 positive[108]. In 2003, Wiestner *et al* and Crespo *et al* showed that the expression of ZAP-70 in CLL has correlated well with IGHV mutation (unmutated *IGHV* genes), disease progression and survival and also the expression of ZAP-70 showed to be quite stable over the course of the disease[141,142] which was confirmed by subsequent sequential studies[143-145]. In another study, IGHV unmutated CLL cases with high ZAP-70 expression were frequently present with other cytogenetic features conveying poor prognoses such as deletion 17p, 11q or V3-21 expression[146]. ZAP-70 by itself can be an independent prognostic marker and CLL patients who express both ZAP-70 and CD38 markers seem to be in a high-risk category, whereas the patients of these markers are negative have a good prognosis and discordant cases fall in an intermediate-risk category[147,148]. Also, ZAP-70 methylation was shown to be a significant prognostic indicator for CLL where low ZAP-70 methylation had significantly shortened time to first treatment and overall survival[149].

Methylation and gene expression correlating prognostic markers in CLL

ANGPT2

ANGPT2 (Angiopoietin 2), which plays an important role in angiogenesis is mainly secreted by endothelial cells at sites of active remodeling in an

autocrine manner[150]. ANGPT2 is elevated in many solid tumors and hematological malignancies while correlating with poor prognosis[151-154]. In CLL, the prognostic importance of ANGPT2 is well studied both on expression and methylation, in which high expression levels were found to be correlated with poor prognosis, predicting a shorter overall and first treatment survival. Both 24K and 450K genome-wide methylation array studies implicated *ANGPT2* as one of the most significantly differentiated genes between IGHV prognostic subgroups[155,156]. ANGPT2 methylation correlated inversely with its mRNA expression levels and low ANGPT2 methylation status was associated with adverse prognostic markers and shorter overall survival[157] suggesting ANGPT2 methylation also to be a good prognostic marker.

LPL

Lipoprotein lipase (LPL) has a pivotal role in lipid metabolism by catalyzing the hydrolysis of chylomicrons and very-low-density lipoproteins and also acts as a bridging protein between cell surface proteins and lipoproteins[158].The expression of LPL in CLL B-cells has been related to fatty acid degradation and signaling functional pathways, which may influence CLL biology and clinical outcome[159]. LPL was identified initially as one of the most differentially expressed genes reported in the gene expression profiling studies by Rosenwald and Klein[108,109] suggesting it as an independent prognostic marker also based on other studies too[160-162]. Epigenetic mechanisms have shown that LDL expression is associated with DNA demethylation of the *LPL* gene in unmutated CLL cases and this expression is dependent on microenvironment signals[163,164] suggesting LPL methylation itself acts as an independent prognostic marker in CLL.

Numerous factors including characteristics of the patient (gender, age, performance status, co-morbidity), the disease (burden, kinetics and biology of the tumor) and as well as the sensitivity of the disease to treatment are to be considered for prognosis of a given patient based on complex relationships.

1.2.2 Mantle cell lymphoma

The term 'Mantle cell lymphoma' (MCL) is named as such since it is derived from a subset of naïve pre-germinal center cells localized in primary B follicles or in the mantle region of secondary follicles[165]. MCLs are characterized by different cytological variants: a classical one shows histologically homogeneous populations of small- to medium-sized lymphoid cells including with irregular nuclei, inconspicuous nucleoli, and scant pale cytoplasm[166,167]. In the blastoid cases, the MCL cells have with rounded nuclei, finely dispersed chromatin and inconspicuous nucleoli and whereas in pleomorphic MCL, cells are larger with irregular and pleomorphic nuclei and distinct small nuclei. The most aggressive clinical behaviour with higher proliferation rates and complex karyotypes are observed in blastoid and pleomorphic cases[168] (Figure 10). MCL represents 5-10% of all Non-Hodgkin lymphomas (NHL) cases and considered as one of the most aggressive lymphoid neoplasms with shorter survival rates with a median of 3-5 years[169]. MCL predominately affects elderly individuals (male: female ratio, 3:1) with a median age at diagnosis of 65 years[170].

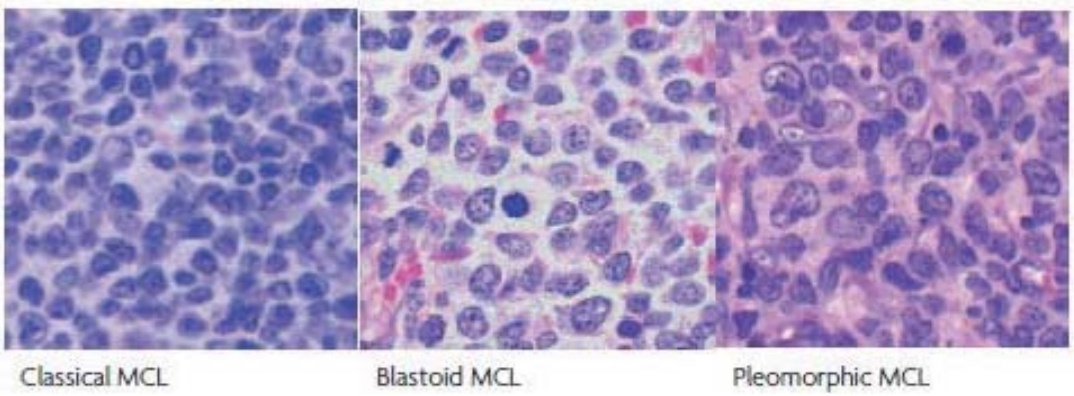

Figure 10: *Histological variants of MCL. Reproduced with permission [168] (Copyright: Nature Reviews Cancer)*

MCL characteristically express monotypic surface IgM and IgD; B-cell antigens CD 19, CD20, CD22; T-cell antigen CD5 and the germinal center-associated antigen CD10[167,171]. CD23 which is also a cell surface marker for B-cell activation and growth do not express in MCL[168]. One important tendency of MCL is that the cells disseminate throughout the body with most patients acquiring lymphadenopathies at diagnosis, but the involvement of the spleen, liver, peripheral blood, bone marrow and the gastrointestinal tract are also very common. Tumors may infiltrate any tissue including the central

nervous system and respiratory tract during the progression of the disease. The genetic hallmark of this neoplasm is the t(11;14)(q13;q32) translocations which involves the Ig heavy chain locus and the BCL-1 oncogene leading to the overexpression of cyclin D1, which has an important pathogenic role, likely deregulating cell cycle control by overcoming the suppressor effect of retinoblastoma 1 (RB1) and the cell cycle inhibitor p27[47,172].

2 SPECIFIC AIMS

1. To study the functional role of MCPH1 in regulation of *ANGPT2* in CLL (Paper I)

2. To study the role of epigenetic silencing of miR26A1 and its effect on EZH2 expression in CLL and MCL (Paper II)

3. To study the functional role of gene-body hypermethylation in gene activation using TET1 as a model system in CLL (Paper III)

3 PATIENTS AND METHODS

My thesis research work has been performed on both patient samples and leukemic cell lines. More detailed materials and methods are provided in the attached papers; hereby the detailed method principles are discussed.

3.1 Patient materials

CLL patient samples used in all three studies were diagnosed according to recently revised iwCLL criteria[102] and peripheral blood mononuclear cell (PBMC) from patients with more than 70% of leukemic cells were selected and collected for the study. The CLL patient samples were collected at the Sahlgrenska University Hospital, Sweden, from the biobank at Uppsala University Hospital, Sweden, and University Hospital, Brno, Czech Republic. Other than CLL patient samples, MCL patient samples were used in paper II. MCL patient samples were diagnosed according to the WHO classification[173] and collected from the biobank at Karolinska University Hospital, Huddinge, Sweden and also from the Department of Molecular Medicine, University of Pavia, Italy. The percentage of Ki-67 staining with a 25% cutoff was used to classify MCL into high proliferation and low proliferation (LP). For normal healthy controls, $CD19^+$ sorted B-cells were isolated from 6 age-matched healthy buffy coats (range: 62–75 years).

3.2 Cell lines and culture conditions

Multiple cell lines were used in these studies. Two CLL (HG3 & MEC1), two MCL (GRANTA519 & Z138) cell lines were used in all the three papers. One Burkitt lymphoma (RAMOS) in the paper I and one breast adenocarcinoma (MCF-7) in the paper I and III were also used. CLL and MCL cell lines were cultured in RPMI-1640 media in the presence of 10% fetal bovine serum, 1x penicillin/streptomycin and 100 mM and 200 mM L-Glutamine respectively. The MCF-7 cell line was used for siRNA transfections, ChIP assays and EMSAs which were cultured in the presence of Dulbecco's Modified Eagle media supplemented with 10% heat-inactivated FBS and 1x penicillin-streptomycin.

3.3 Gene expression analysis by real-time quantitative PCR

mRNA gene expression analysis was performed using real-time quantitative PCR (qPCR). In my studies, both SYBR green and TaqMan based qPCR were performed to analyze the gene expression in both cell lines and patient samples. The basic principle of qPCR includes at first total RNA is transcribed into complementary DNA (cDNA) with the help of an enzyme called reverse transcriptase enzyme. The resultant cDNA is then used as the template for the qPCR reaction for analyzing gene expression. Primers were designed by using Primer 3 software for SYBR green based qPCR assay, whereas custom labelled ready to use TaqMan primers were used for TaqMan based qPCR assay. ΔΔCt method used for analyzing the expression of a gene of interest and the differences in expression were calculated with the help of a control gene.

3.4 Protein expression analysis by western blot

In the publications, assessment of protein levels was performed by western blot assay. Western blotting is a biochemical technique used to identify specific proteins in a complex sample mixture which was first described in 1979[174]. The basic principle in western blotting is that the proteins are separated by size using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a membrane by electroblotting. The membrane is then treated with blocking solution (5% BSA in our study) and then probed sequentially with primary and secondary antibodies to detect proteins of interest. In all the three papers, western blotting was performed with equal amount of total cell lysates and in some cases nuclear lysates using RIPA buffer with PI inhibitors were loaded into Bis-Tris gels and transferred to Hybond ECL membranes. The membranes were then treated with 5% BSA in TBS with the addition of 0.1% Triton X-100. After blocking, the membranes were incubated with the appropriate primary and secondary antibodies, followed by washes with TBS containing 0.05% Triton X-100. Blots were visualized with SuperSignal West Dura Extended Duration Substrate using the ChemiDoc XRSC instrument.

3.5 Methylation analysis using with pyrosequencing

Quantitative measurement of methylation was performed with pyrosequencing. The pyrosequencing technique is based on a sequencing-by-synthesis principle[175] in which a DNA segment (bisulfite converted DNA) is

amplified and the strand to serve as the pyrosequencing template is biotinylated. The biotinylated single-stranded PCR amplicon after denaturation is isolated and allowed to hybridize with a respective sequencing primer. Four enzymes namely DNA polymerase, ATP sulfurylase, luciferase, and apyrase, as well as the adenosine 5' phosphosulfate (APS) and luciferin substrates are incubated with the single-strand template and hybridized primer in order to precisely detect nucleic acid sequences during the synthesis. At first, DNA polymerase catalyzes the addition of four deoxyribonucleotide triphosphate (dNTP) to the sequencing primer if it is complementary to the base in the template strand. Pyrophosphate (PPi) releases after each incorporation event in a quantity equimolar to the amount of incorporated nucleotide. This PPi converts into ATP by ATP sulfurylase in the presence of APS at which the ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light which is proportional to the amount of ATP consumed. This visible light produced by this reaction is seen as a peak in the pyrogram output. Following this, nucleotide-degrade enzyme, Apyrase, degrades continuously the unincorporated nucleotides and also ATP. This process is continued and eventually, the complementary DNA strand is built up and the sequence is determined from the signal in the pyrogram where the percentage of methylation is noted[176,177].

For analyzing methylation levels, at first, the genomic DNA was bisulfite converted. Three primers, namely forward, reverse (one among this primer was biotin labelled) and sequence sequencing primer is designed with the help of pyro mark assay design software. The bisulfite converted DNA was amplified with forward and reverse primers. The amplified PCR product was immobilized with streptavidin sepharose high-performance beads followed by annealing with sequencing primer. The analysis was then performed using the pyrosequencer instrument and the CpG site methylation percentage of methylation was calculated for all CpG sites in the target sequence.

3.6 Electrophoretic mobility shift (EMSA) assay

EMSA is a sensitive and rapid method to detect DNA binding proteins was developed as a method in 1981[178]. In this technique, DNA-protein complexes migrate slower than non-bound DNA in a native polyacrylamide or agarose gel, resulting in a “shift” in the migration of the labelled DNA band[179]. Here

we used this method for the detection of transcription factors and other sequence-specific DNA binding proteins.

In my first paper, we performed EMSA in which at first the nuclear extracts from siRNA-transfected MCF-7 cells and from the PBMCs of CLL patients were prepared. Equal amounts of nuclear extract protein were incubated with poly (dIdC), 32P-labeled oligonucleotide probe/biotin-labelled ANGPT2 probe (with and without a possible MCPH1-binding site), and 1x binding buffer. Following this, the samples were incubated on ice, and run on 6% DNA retardation gels. Membranes were cross-linked with UV, blocked and conjugated followed by incubation of MCPH1 antibody. The radioactive gels were analyzed directly by phosphorimager analysis, and whereas biotin labelled gels were developed with an equipped CCD camera ChemiDoc XRSC instrument.

3.7 Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) is a well-known and common technique for investigating specific protein–DNA interactions and also become one of the most practical and useful techniques to study the mechanisms of gene expression, histone modification, and transcription regulation. The principle behind the ChIP assay included to fix the protein-DNA complex in living cells and then randomly sheared into 100-500 bp DNA fragments by sonication or nuclease digestion methods to selectively enrich the DNA fragments fixed with targeted protein by using ChIP-grade antibodies followed by purification before using in downstream analysis[180-182].

In the paper I and III, ChIP was performed on the transfected cells which were crosslinked with formaldehyde, lysed and sonicated four times for 10min each. The sonicated complex was incubated with appropriate ChIP-grade antibodies and purified. The final precipitated DNA was analyzed by SYBR green-based real-time quantitative PCR. ΔΔCt method from the Excel-based ChIP-qPCR analysis template was used for the calculation of the normalized percentage of input and fold enrichment values for ChIP data.

3.8 Measuring the promoter activity by Luciferase reporter assay

Luciferase and β-galactosidase assays were used to study gene expression as well as other cellular components and events that are involved in gene

regulation. This assay is extremely rapid, simple, sensitive, and possesses a broad linear range. Due to its high sensitivity, even small changes in transcription can be quantified. The principle of this luciferase reporter assay includes the use of luciferases which are oxidative enzymes that convert luciferin into oxyluciferin in the presence of oxygen by which the energy released measured in the form of visible light or bioluminescence. At first, the regulatory region of an interested gene cloned in the luciferase expression vectors which then introduce the resulted vector DNA transfected into cells and then allow the cells to grow over a period of time. The transfected cells were lysed and measured using a luminometer in the presence of luciferin and necessary cofactors. The resultant light from lysates gives quantitative reading by which the luciferase activity can be directly correlated the activity with the gene of interest[183].

$$\text{Luciferein} + O_2 + \text{ATP} \xrightarrow[\text{Luciferase}]{} \text{Oxyluciferin} + CO_2 + \text{AMP} + \text{PPI} + \text{LIGHT}$$

In the paper I, MCPH1 and ANGPT2 promoters and in paper III, in order to identify the HMR promoter activity and downstream cryptic region, the sequences were cloned into a vector and PCR-amplified. These amplified promoter sequences were cloned into the respective basic Luciferase vector and transiently transfected into MCF-7 cells in the presence of a β-galactosidase reporter gene. Luciferase activity was determined 24 h and 48 h after transfection by use of the dual-luciferase reporter assay system in duplicate samples manually. The emitted relative light units were measured with a luminometer and the final luciferase values (relative light units) were calculated by dividing the luciferase activity by the β-galactosidase activity.

3.9 Analysis of protein-protein binding affinities by Co-immunoprecipitation (Co-IP)

Co-IP is widely used to study protein-protein binding affinities by using specific antibodies to indirectly capture proteins that are confining to a specific target protein. In a co-IP, at first the antibody against a target protein is coupled to sepharose beads and the complexes containing the target protein are immunoprecipitated by centrifugation with antibody coupled beads. The targeted protein components in the complexes are visualized by western blotting using specific antibodies to the different components[184].

In Paper I, co-IPs have been performed to check the binding affinities of MCPH1, E2F1, DNMT1 and DNMT3b proteins by using sepharose G beads and followed western blotting to detect the specific binding affinities of these proteins.

3.10 Assessment of apoptosis and expression through flow cytometry (FACS)

Flow cytometry is a powerful tool that utilizes multiparametric laser-based-technology to analyze the physical characteristics of single cells and count, sort and profile cells in a heterogeneous fluid mixture. FACS determines the phenotype, function and sorts the cells according to these parameters. The principle behind FACS include the cells or other substances suspended in a liquid stream mixture being passed through a laser beam one at a time, by which the interaction of the light is measured as light scatter and fluorescence intensity. The specific cellular component is bound to the defined fluorochrome by which the fluorescence intensity will ideally represent the amount of that specific cell component[185,186]. FACS analysis was performed in the paper II to measure apoptosis by Annexin V and EZH2 expression levels and data analyzed using the FACSDiva software. We followed the kit protocol for analyzing the apoptosis and to analyze the EZH2 expression levels. Cells were permeabilized with solution B followed by incubation with diluted EZH2 primary antibody and APC conjugated goat anti-rabbit IgG secondary antibody.

3.11 Statistical analysis

All statistical analyses were performed with SPSS, STATISTICA and GraphPad Prism software. In the paper I, correlations between *MCPH1* variants, *ANGPT2* and *hTERT* were made and in paper II the correlation of expression levels of *miR26A1* and *EZH2* were assessed by Spearman's two-tailed correlation analysis. All the comparisons between groups were performed with a two-tailed Student's t-test in the papers. For the assessment of overall survival, the Kaplan–Meier survival analysis with a log-rank test was performed.

4 RESULTS & DISCUSSION

4.1 MCPH1 maintains long-term epigenetic silencing of *ANGPT2* in chronic lymphocytic leukemia (Paper I)

Microcephalin (*MCPH1*) is a well-known tumor suppressor, implicated in DNA damage response, cell cycle regulation, X-chromosome inactivation in development and chromosome condensation, and whereas *ANGPT2* is an oncogene[157] involved in angiogenesis and having a major role in tumor growth and metastasis. *ANGPT2* is located on the same chromosome as *MCPH1* and overlaps with *MCPH1* in the opposite orientation. In this paper, we wanted to study the functional role of MCPH1 in regulating *ANGPT2*, along with hTERT which is a direct target of *MCPH1*. Both *ANGPT2* and *hTERT* show differential expression and are regulated epigenetically by DNA promoter methylation. Although the prognostic importance of ANGPT2 and hTERT in CLL has been shown earlier, the prognostic value of MCPH1 in CLL subsets has not been studied previously. In this study, using Spearman correlation analysis, the expression level of *MCPH1* was shown to be significantly inversely correlated with the expression of *ANGPT2* and *hTERT*. The mRNA expression levels of *MCPH1, ANGPT2* and *hTERT* showed statistically significant differences between CLL prognostic subgroups IGHV mutated and IGHV unmutated (*MCPH1*, P=0.007; *ANGPT2*, P=0.0002; *hTERT*, P<0.0001) based on the qPCR analysis. When *MCPH1* was downregulated in CLL cell lines, we observed upregulation of *ANGPT2*, accompanied by loss of its promoter methylation, implicating the role of MCHP1 in *ANGPT2* regulation. Here we showed mechanism behind MCPH1 mediated regulation of *ANGPT2* promoter, either through promoter competition model (as both promoters contain E2F1- binding sites and have been shown to be induced by increasing concentrations of E2F1) or silencing of the promoter in cis (as shown by *MCPH1*-mediated silencing of hTERT promoter activity by binding to its promoter). Later, using ChIP assay and Co-IPs, we also noticed that MCPH1 binds to the *ANGPT2* promoter and recruits DNA methyltransferases, thereby silencing *ANGPT2* in IGHV mutated CLL samples where MCPH1 is expressed in higher levels compared to IGHV unmutated samples (Figure 11).

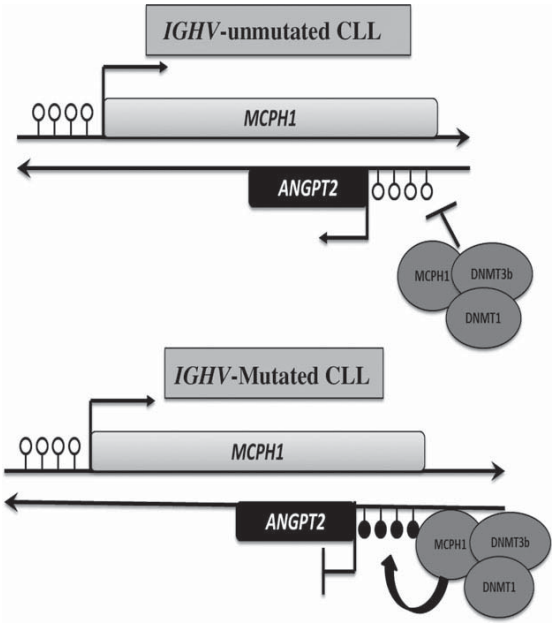

Figure 11: *Model explaining the role of MCPH1 in regulating ANGPT2 expression in both IGHV-unmutated and IGHV-mutated CLL patient samples in vivo.*

According to our results, along with *ANGPT2* and *hTERT*, the *MCPH1* expression could be a possible prognostic marker in CLL. *MCPH1* has three variants namely *MCPH1* full length, *MCPH1* Sh1 and *MCPH1* Sh2. Here, we studied the role of *MCPH1* full length and Sh2 but not Sh1 as this variant has complete sequence homology which cannot be distinguished with other variants. In conclusion, we showed a novel function of *MCPH1* in epigenetic silencing of the *ANGPT2* promoter by interacting with and recruiting DNMTs to this promoter in CLL and with these results, our study unravels reason behind the differential methylation status of *ANGPT2* in CLL.

4.2 Epigenetic silencing of miR-26A1 in chronic lymphocytic leukemia and mantle cell lymphoma: Impact on EZH2 expression (Paper II)

In line with earlier published studies, our study also reported downregulation of *miR26A1* in CLL as shown in a variety of B-cell malignancies with lower expression levels in CLL patient samples compared to normal B-cells suggesting that *miR26A1* acts as tumor suppressor microRNA[47,155,187-189]. On

the other hand, EZH2 which is shown to be overexpressed in CLL is known to silence gene promoter either directly using histone methyltransferase activity or through DNA methylation machinery. According to our earlier published 450K DNA methylation array data, *miR26A1* was differentially hypermethylated between CLL prognostic groups. We also performed 450K methylation arrays in MCL patient samples and found that *miR26A1* was significantly hypermethylated in all samples compared to normal healthy controls. To validate the findings of 450K DNA methylation array data in CLL and MCL, we investigated the molecular role of *miR26A1* and also its target EZH2, using a large cohort of CLL and MCL samples. Based on our pyrosequencing and RT-PCR analysis, *miR26A1* was hypermethylated and poorly expressed in CLL and MCL samples compared to normal healthy B-cells suggesting that the above results clearly support with 450K DNA methylation array data. Moreover, the percentage of *miR26A1* methylation predicts risk stratification in CLL, where hypermethylation correlates with poor prognosis and shorter survival. In order to investigate the functional role of *miR26A1* in regulating its target EZH2, we have overexpressed *miR26A1* mimic microRNA and negative control mimic microRNA in CLL and MCL cell lines using Amaxa Nucleofection. Immunoblotting and FACS labelling showed reduced EZH2 protein levels in all cell lines. Furthermore, to know the correlation between *miR26A1* and EZH2, we first selected CLL primary samples exhibiting very high (10 samples out of 70 samples) and low (10 samples) expression levels in *miR26A1* and analyzed the *EZH2* mRNA expression levels using RT-PCR. There was a significant negative correlation between *miR26A1* and *EZH2* (P=0.04, r= -0.45). Increased apoptosis was observed when *miR26A1* was overexpressed in CLL and MCL cell lines, followed by a decrease in EZH2 protein levels which was analyzed by FACS. In CLL, the more varying degree of *miR26A1* methylation was noted, with high methylation predicting a significantly shorter survival, a finding that needs further studies. Thus our data showed that *miR26A1* was epigenetically regulated by DNA hypermethylation in CLL and MCL primary samples compared with normal samples. With these results, we conclude a tumor suppressor role of *miR26A1* in CLL and MCL, which is regulated by DNA hypermethylation. Overall, with our results, we suggest that EZH2 could be one of the therapeutic targets in CLL.

4.3 Gene-body hypermethylation controlled cryptic promoter and miR26A1-dependent EZH2 regulation of TET1 gene activity in chronic lymphocytic leukemia (Paper III)

The Ten-eleven-translocation 1 (TET1) is differentially expressed in leukemia as in many other cancers. However, the mechanism behind TET1 gene regulation is still not known. Gene-body hypermethylation studies recently point out a transcriptional regulatory step in controlling alternative splicing and cryptic promoter[190,191]. Earlier, using MBD seq[192] for DNA methylation profiling between CLL patient samples and normal B-cell controls we found that *TET1* was one of the significantly differentially methylated genes with gene-body hypermethylation. Here, we wanted to understand the role of gene-body hypermethylation in *TET1* gene expression as gene-body hypermethylation was shown to be correlated with gene activation. We characterized the mechanisms that control *TET1* gene activity at the transcriptional level by using both patient and cell line samples. We validated the expression and methylation status of TET1 using 40 CLL patient samples and two different CLL cell lines. Our data showed that there is a significant differential expression of *TET1* in CLL compared with normal control samples which also supported the analysis performed by us with independently published RNA seq data[193]. Our study showed that unlike promoter methylation, gene-body hypermethylation of *TET1* positively correlated with *TET1* gene expression in CLL samples. According to our earlier study in CLL[58] *miR26A1* was shown to be promoter hypermethylated and silenced, so we treated CLL cell lines with 5-aza-2'-deoxycytidine (DAC) which resulted in the activation of *miR26A1* (which was shown to directly target EZH2), resulting in a decrease in both mRNA and protein levels of EZH2. Based on ChIP assays we noticed that *miR26A1* can regulate *TET1* at the transcriptional level through negatively correlating with EZH2 expression levels, illustrating the functional interplay between *EZH2*, *TET1* and *miR26A1*. These further results in the decreased occupancy of EZH2 over the *TET1* promoter and consequently exhibits the loss of TET1 expression. Moreover, we identified the presence of cryptic promoter on *TET1* hypermethylated gene-body based on both strand-specific RT-PCR and promoter luciferase assay, suggesting that cryptic promoter encodes antisense transcripts. Furthermore, we found that antisense cryptic transcription may be regulating *TET1* gene expression in part by occluding the transcription initiation machinery from the *TET1* promoter. We showed a novel correlation

between increased expressions of intronic transcripts with decreased *TET1* promoter activity through the loss of RNA Pol II occupancy (Figure 12). Further studies are needed to characterize the functional role of the identified cryptic transcript in CLL.

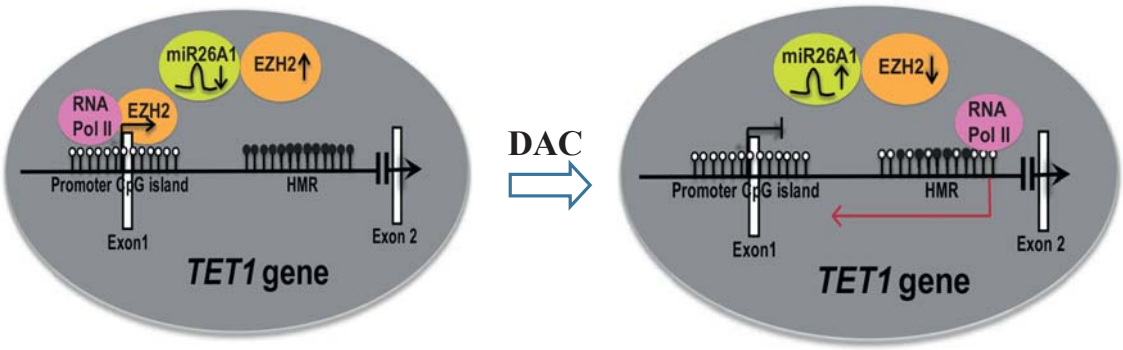

Figure 12: *Model explaining the role of DNA hypermethylation in regulating TET1 gene expression.*

5 CONCLUSION

My thesis is about understanding the epigenetic mechanisms involved in regulating tumor suppressors and oncogenes which are selected as significantly differentially methylated in CLL samples compared to normal healthy controls using global DNA methylation profiling studies. For the validation studies, independent large cohorts of CLL patient samples are used for the gene expression and methylation analysis. In addition to CLL samples to unravel the epigenetic mechanisms of these tumor suppressors and oncogenes, we used CLL and MCL cell lines as a model system. My work has shown the DNA methylation has a prominent role on regulating the gene expression in CLL and also the percentage of DNA methylation can be used as an independent prognostic marker along with existing markers such as CD38, ZAP70, IGHV mutational analysis etc.., which also correlates with overall survival in CLL patient samples.

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