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Array-based Characterization of Chronic Lymphocytic Leukemia

*- with Focus on Subsets Carrying Stereotyped B-cell
Receptors*

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Abstract

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In chronic lymphocytic leukemia (CLL), the presence of multiple subsets expressing 'stereotyped' B-cell receptors (BCRs) has implicated antigen(s) in leukemogenesis. These stereotyped subsets display similar immunoglobulin (*IG*) gene usage, almost identical complementarity determining region 3's and may share clinical features. For instance, subsets #1 (*IGHV1/5/7/IGKV1-39*) and #2 (*IGHV3-21/IGLV3-21*) have inferior outcome compared to non-subset patients, whereas subset #4 (*IGHV4-34/IGKV2-30*) display a favourable prognosis. The aim of this thesis was to investigate genomic aberrations, gene expression patterns and methylation profiles in stereotyped subsets and compare epigenetic profiles in CLL and mantle cell lymphoma (MCL).

In **paper I**, we investigated genomic aberrations in subsets #2, #4 and #16 and in non-stereotyped samples (n=101) using high-density 250K SNP arrays. Subset #2 and non-subset #2 *IGHV3-21* cases displayed a higher frequency of aberrations than subset #4 cases. The high incidence of del(11q) in both subset #2/non-subset #2 may reflect the adverse survival reported for *IGHV3-21* patients. In contrast, the lower frequency of genetic events and lack of poor-prognostic aberrations in subset #4 may partially explain their indolent disease. In **paper II**, we analysed the global RNA expression in subset #4, #16 and non-subset *IGHV4-34* CLL patients (n=25). Subsets #4 and 16 showed distinct gene expression profiles, where genes involved in cell regulatory pathways were significantly lower expressed in subset #4, in line with their low-proliferative disease. In **paper III**, a genome-wide methylation array was applied to investigate methylation profiles in subsets #1, #2 and #4 (n=39). We identified differential methylation patterns for all subsets and found affected genes to be involved in e.g. apoptosis and therapy resistance. When performing functional annotation, a clear enrichment of genes involved in adaptive immunity was observed. These genes were preferentially methylated in subset #1 when compared to either subset #2 or #4, possibly due to different antigen responses. In **paper IV**, the genome-wide methylation profiles for 30 CLL and 20 MCL patients were investigated. Distinct methylation profiles were observed, where MCL displayed a more homogeneous profile. Homeobox transcription factor genes showed a higher degree of methylation in MCL, while apoptosis-related genes and proliferation-associated genes were methylated in CLL.

In summary, this thesis demonstrates that stereotyped CLL subsets display differences in gene expression profiles, genetic aberrations and methylation patterns, underscoring the functional relevance of subgrouping according to BCR stereotypy. The distinct methylation profiles of CLL and MCL suggests that different epigenetic mechanisms are involved in the pathogenesis of these B-cell malignancies.

Keywords: chronic lymphocytic leukemia, array-based characterization, stereotyped B-cell receptors, subsets, antigens, SNP array, gene expression array, methylation array

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Tamdiu discendum est, quamdiu vivas (Séneca)

“There is a need for learning, for as long as you may live”

Till min kära familj

List of Papers

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

- I **Marincevic M***, Cahill N*, Gunnarsson R*, Isaksson A, Mansouri M, Göransson H, Rasmusson M, Jansson M, Ryan F, Karlsson K, Adami HO, Davi F, Jurlander J, Juliusson G, Stamatopoulos K, Rosenquist R. High-density screening reveals a different spectrum of genomic aberrations in chronic lymphocytic leukemia patients with "stereotyped" *IGHV3-21* and *IGHV4-34* B cell receptors. *Haematologica*. 2010 Sep;95(9):1519-25
- II **Marincevic M***, Mansouri M*, Kanduri M, Isaksson A, Göransson H, Ekström Smedby K, Jurlander J, Juliusson G, Davi F, Stamatopoulos K, Rosenquist R. Distinct gene expression profiles in subsets of chronic lymphocytic leukemia expressing stereotyped *IGHV4-34* B cell receptors. *Haematologica*. 2010 Aug 26. [Epub ahead of print].
- III **Marincevic M***, Kanduri M*, Halldorsdottir AM, Mansouri M, Göransson H, Isaksson A, Juliusson G, Ehrencrona H, Stamatopoulos K, Rosenquist R. Chronic lymphocytic leukemia subsets with stereotyped B-cell receptors are distinguished by unique methylation patterns. Manuscript.
- IV Halldorsdottir AM*, Kanduri M*, **Marincevic M**, Mansouri M, Isaksson A, Göransson H, Stamatopoulos K, Sander B, Ehrencrona H, Rosenquist R. Genome-wide array-based methylation profiling reveals preferential methylation of homeobox transcription factor genes in mantle cell lymphoma and pro-apoptotic genes in chronic lymphocytic leukemia. Manuscript.

*Contributed equally to this work.

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Other publications

- V **Marincevic M**, Tobin G, Rosenquist R. Infrequent occurrence of *PIK3CA* mutations in chronic lymphocytic leukemia. *Leukemia & Lymphoma* 2009 May;50(5):829-30.

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Abbreviations

AID	Activation induced cytidine deaminase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATM	Ataxia telangiectasia mutated
BCR	B-cell receptor
C	Constant
CD	Cluster of differentiation
CDR	Complementarity determining region
CLL	Chronic lymphocytic leukemia
CMV	Cytomegalovirus
CNA	Copy number aberration
CNN	Copy number neutral
CNV	Copy number variation
D	Diversity
DLBCL	Diffuse large B-cell lymphoma
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FR	Framework region
EBV	Epstein-Barr virus
FISH	Fluorescence <i>in situ</i> hybridization
GC	Germinal center
GEP	Gene expression profiling
GOTM	Gene ontology tree machine
IG	Immunoglobulin
IGH	Immunoglobulin heavy
IGK	Immunoglobulin kappa
IGL	Immunoglobulin lambda
IL	Interleukin
IPA	Ingenuity pathway analysis
J	Joining
LOH	Loss of heterozygosity
LPL	Lipoprotein lipase
Mbp	Mega basepair
MBL	Monoclonal B-cell lymphocytosis
MCL	Mantle cell lymphoma
MI	Methylation index
MM	Multiple myeloma

NAL	N-acetyllactosamine
NK	Natural killer
oxLDL	Oxidized low-density lipoprotein
PCA	Principal component analysis
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RQ-PCR	Realtime quantitative PCR
SHM	Somatic hypermutation
SNP	Single nucleotide polymorphism
UPD	Uniparental disomy
V	Variable
WHO	World health organisation
ZAP70	Zeta-chain-associated protein kinase 70

INTRODUCTION

Today, many types of lymphomas and leukemias of lymphatic origin have been identified, each constituting an individual and heterogeneous disease with regards to both biological and clinical features. Such tumors are derived from cells that normally function in the immune system, i.e. B-cells, T-cells, or natural killer (NK) cells¹, however the majority are of B-cell origin. During the development of a lymphoid cell progenitor, the cell undergoes several dangerous genetic events which may disrupt the regulatory mechanisms that drive and control cell proliferation, differentiation and apoptosis and thus result in transformation into a tumor cell².

In this thesis, the focus will be on one of the most common B-cell lymphomas, namely B-cell chronic lymphocytic leukemia (CLL). Here, we have performed high resolution array-based studies to increase our knowledge of genetic and epigenetic events in recently described CLL subset with “stereotyped” B-cell receptors (BCRs) and distinct clinical outcome. A key molecule towards the understanding of CLL is the BCR, which may provide valuable hints to the pathogenesis of the disease. Therefore, a basic introduction into B-cell development and immunoglobulin (IG) genes is first provided.

B-cell development and immunoglobulin gene rearrangement

Normal B-cell development

B-cells are derived from hematopoietic stem cells and are produced in the bone marrow. During B-cell development, they are dependent upon stromal cell interaction which releases growth factors that promote cell division³. These bone-marrow stromal cells are essential for maturation of the progenitor B-cells, both through their interaction with these progenitor cells as well as secretion of various cytokines which supports their development, e.g. IL-7⁴. The B-cell at the earliest stage is called a pro-B-cell and it is at this stage that the *IG* heavy-chain (*IGH*) locus starts to rearrange and CD19 is expressed^{5,6}. Subsequent to this, the pre-B-cell rearranges one of the *IG*

light chain loci and IgM is expressed. Next, the immature B-cell undergoes a process called negative selection whereby cells recognizing self-antigens will be suppressed or undergo apoptosis⁷. The surviving immature B-cells exit the bone marrow where they undergo further developmental steps, producing IgD in addition to IgM and becoming mature naïve B-cells. These naïve B-cells will start to circulate and recognize foreign antigens giving rise to an activated B-cell that either differentiates into an antibody producing plasma cell or a long-lived memory cell^{5,8}.

Immunoglobulin gene rearrangement

The BCR on the surface of the cell plays a vital role in recognizing foreign antigens. In the body, B-cells expressing a vast array of BCRs circulate in the blood and lymphatic system and perform the role of immune surveillance. One of the most important stages during B-cell development is rearrangement of the *IG* gene loci which encodes the BCR. The IG molecule is a membrane protein complex that consists of two identical heavy chains and two identical light chains (Figure 1). Each heavy chain is joined to a light chain by a disulphide bridge along with non-covalent interactions such as hydrogen bonds, hydrophobic bonds and salt-linkages.

The *IGH* locus is located on chromosome 14 at band 14q32.33, is approximately 1,250 kb long and consists of clusters of 38-46 variable (V) functional genes, 23 diversity (D) genes, 6 joining (J) genes and 9 constant (C) germline genes⁹⁻¹². The *IGHV* genes are divided into seven subgroups (*IGHV1-7*), where the *IGHV3* subgroup is the largest (21 members), followed by *IGHV4* (10 members) and *IGHV1* (9 members)¹³. Furthermore, there are two *IG* light chain loci, *IG* kappa (*IGK*) and *IG* lambda (*IGL*), located on chromosomes 2 and 22, respectively. Unlike the *IGH* locus, the *IG* light chain loci are made up of only V and J genes and lack D genes. Therefore, the degree of light chain diversity is more limited in comparison to the *IGH* locus. Similar to the *IGHV* genes, the *IG* light chains are also subdivided based on similarities in nucleotide sequence, resulting in 7 kappa subgroups (*IGKV1-7*) and 10 lambda subgroups (*IGLV1-10*)¹³.

Recombination of the *IGHV-D-J* genes results in the formation of a functional heavy chain and is also the primary step in the creation of IG diversity. During the process of *IG* gene rearrangement, one *IGHD* and one *IGHJ* gene segment are firstly joined together, followed by rearrangement of one *IGHV* gene to the pre-formed *IGHD-IGHJ* complex⁹. This *IGHV-D-J* rearrangement gives rise to the V region, responsible for antigen binding in the heavy chain molecule (Figure 1). Within this region, there are three areas displaying hypervariability, defined as complementarity determining regions (CDRs). The greatest diversity is found within the CDR3, which contains the V, D and J genes.

Following a successful recombination, similar steps occur at the *IGK/IGL* gene loci, during which an *IGK/LV* gene is joined to an *IGK/LJ* gene segment. During this process, the *IGK* genes will recombine first and if both alleles produce non-functional rearrangements, the *IGL* gene will be utilized instead¹⁴. Both these essential combinatorial events occurring at the *IGH* and *IG* light chain loci generate diversity within the antibody binding site which gives every B-cell a unique *IG* gene sequences ($\sim 2 \times 10^6$ possible combinations). Additionally, throughout the rearrangement process, introduction of N nucleotides into the junction regions and exonuclease activity generates further diversity of the BCR repertoire. Moreover, introduction of somatic hypermutations generates additional variation after VDJ recombination further increasing the antibody diversification (see below). Based on all of these processes, the chance of two B-cells expressing identical BCRs is approximately 1 in 2.3×10^{12} ¹². Subsequently, every single B-cell will express a unique IG molecule on their cell surface with a specific antigen target.

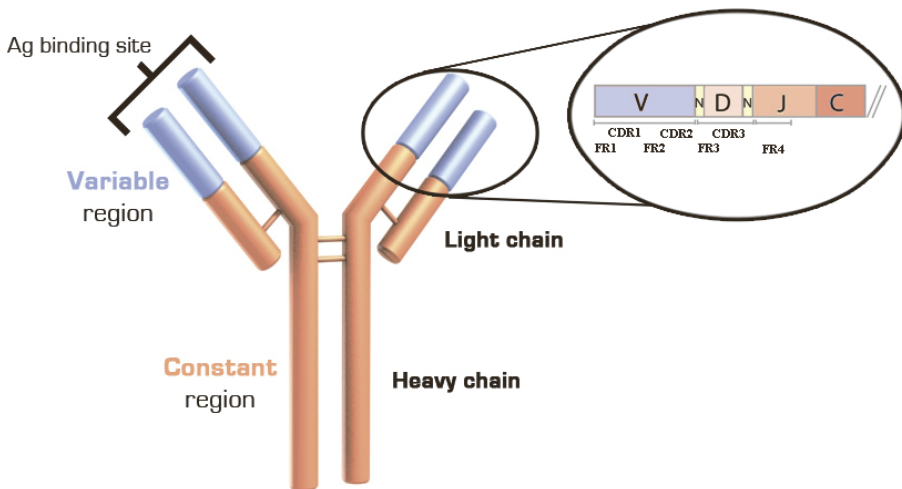


Figure 1: Immunoglobulin structure and V(D)J rearrangement.

Germinal centers and somatic hypermutation

The germinal centers (GC) are sites within lymph nodes in peripheral lymphoid tissue where naive B-cells mainly encounter antigens. This interaction results in the activation and rapid proliferation of the B-cells, but they can also undergo somatic hypermutation (SHM) as well as class switching¹⁵.

The SHM process forms a second round of diversification and is particularly focused within the six CDRs that form the antigen binding site. SHM consists of single base substitutions that occur at a frequency of 10^3 - 10^4 per base per cell division¹⁶. Occasionally, insertions or deletions are also introduced. The initial step of SHM is mediated by the enzyme, activation-induced cytidine deaminase (AID) which removes the amino group from the cytosine in the single stranded DNA resulting in a U:G (U=uridine) mismatch^{17,18}. The AID enzyme is also involved in *IG* class switching where the antibody isotype is changed from IgM to IgG, IgA or IgE. During this process, the *IGHM* gene is deleted and subsequent recombination takes place with another constant gene either *IGHG*, *IGHA* or *IGHE*¹⁹. As the V region does not change, class switching does not affect antigen specificity, thereby retaining the affinity of the antibody for the same antigens.

Since B-cell leukemia and lymphomas arise from a single B-cell carrying unique *IGH* and light chain gene rearrangements, these can be used as clonal markers for tracking the individual tumor. The *IG* gene rearrangements will therefore reflect the tumor precursor cell and its history of antigen exposure, as revealed by the presence or absence of SHMs (discussed further in a later section)²⁰.

Chronic lymphocytic leukemia

Introduction

Over the past decade, considerable progress towards understanding the pathogenesis of CLL has been achieved. This incurable lymphoproliferative disorder is the most common adult leukemia in Western countries with ~500 new patients diagnosed annually in Sweden. CLL is most frequent in elderly people with a median age at diagnosis of approximately 70 years, and is more commonly found in males than females (ratio 2:1)²¹.

CLL is characterized by a monoclonal accumulation of small long-lived neoplastic B-lymphocytes²². The diagnostic criteria include an absolute lymphocyte count of $>5.0 \times 10^9/L$ in peripheral blood, immunophenotypic expression of CD19⁺, CD5⁺, CD23⁺ with low levels of cell surface IgM/IgD, and CD79b, and a typical bone marrow or blood morphology with infiltration of small mature looking lymphocytes²². Many patients are asymptomatic at diagnosis, but some patients may present with enlarged lymph nodes, infections and hemolysis.

CLL is known to be a biologically and clinically heterogeneous malignancy where many patients survive for a long time (10-15 years) without treatment, whereas others succumb rapidly to the disease despite therapy. Among CLL patients, the most common complications observed are infections of the respiratory tract, skin or urinary tract. These are caused by bacterial infection of *Streptococci*, *Staphylococci* or *Escherichia* and are the major cause of mortality among CLL patients^{23,24}.

Etiology

Currently, the cause of CLL remains unknown. However, several studies have noted that first-degree relatives of CLL patients carry a higher risk of developing CLL (7-8.5 fold increase), hence suggesting a shared genetic susceptibility for familial CLL. Moreover, family members of CLL patients may also be at increased risk of developing other hematologic malignancies²⁵⁻²⁷. Furthermore, studies have found evidence for genetic susceptibility in CLL at for instance 2q37.3, 8q24.21, 15q21.3 and 16q24.1 and hence identify low-risk variants predisposing to CLL²⁷⁻³⁰. Recent studies have shown that the presence of monoclonal B-cell lymphocytosis (MBL) may represent a pre-clinical stage of CLL since MBL may share the same immunophenotype as CLL. Furthermore, it was demonstrated that 3.5% of adults with normal blood counts exhibited MBL that presented the same phenotype as CLL³¹. Although only 1% of MBLs transform to CLL, it has been proposed that MBL may be interpreted as a pre-stage disease to CLL and that chronic and persistent antigenic stimulation may lead to progression into CLL³². In line with this, several studies have provided evidence for antigen involvement in CLL and that CLL may occur following a response to an infectious agent (described later). It has also been shown that respiratory tract infections such as pneumonia may be a potential CLL trigger but also represent a premalignant immune disruption preceding CLL³³.

Cell of origin

Today, several observations have been made that strongly implicate antigen recognition and antigen selection in the development of CLL. So from which cell population do CLL clones arise? In the late 1980's it was hypothesised that CLL originated from a pre-GC cell^{34,35}, however, successive reports found that in half of all CLL cases the *IGHV* genes were mutated³⁶⁻³⁹, hence separating CLL patients into two entities. These findings suggested that CLL cases with *IGHV* unmutated genes originated from a pre-GC cell, while those with *IGHV* mutated genes possibly arose from a post-GC compartment. However, gene expression profiling (GEP) studies by Rosenwald *et al* and Klein *et al* in 2001 illustrated that *IGHV* mutated and

unmutated CLL displayed a similar profile^{40,41}. Furthermore, it was found that CLL cells appeared to display a signature similar to memory cells⁴⁰. In addition, characterization of cell surface markers in *IGHV* mutated and unmutated CLL again confirmed the idea that CLL arose from an antigen experience origin⁴².

Treatment

In CLL, a ‘watch and wait’ approach is generally employed for the majority of patients, particularly where a clinically indolent course is observed. Hence, asymptomatic patients at an early stage of disease are monitored without therapy²². However, among untreated patients at an early stage of disease, 40% will progress to an advanced disease and 45% will ultimately require therapy⁴³. The first-line treatment consists of a combination of fludarabine and cyclophosphamide. This combination results in ~95% overall response rate and ~25% complete remission⁴⁴. In elderly and weak patients, a low dose treatment using low toxicity agents such as chlorambucil is the treatment of choice⁴⁴. Furthermore, the combination of fludarabine, cyclophosphamide, and rituximab treatment for CLL patients has been shown to improve survival in patients with relapsed-refractory disease and recently implemented as a first line treatment⁴⁵⁻⁴⁷. Another treatment option includes the use of monoclonal antibodies against CD52 (Campath 1H/Alemtuzumab), particularly in fludarabine-resistant/17p deleted cases⁴⁸. Stem cell transplantation is generally reserved for younger patients with unfavorable risk factors⁴⁹.

Prognostic markers

Clinical staging systems

Today, two clinical staging systems are used in CLL, namely Binet (stage A-C) and Rai (stage 0-IV). These two staging systems divide patients into risk groups based on evaluations of clinical findings regarding the disease burden⁵⁰⁻⁵². CLL patients in Rai stage 0 or Binet stage A normally survive beyond 10 years, while patients in Rai stage I/II or Binet stage B have a median survival of 5-7 years. Patients in the worst group, Rai stage III/IV or Binet stage C have a median survival of less than three years⁵³. However, these staging systems do not accurately predict which patients will develop progressive disease at an early stage.

IGHV gene mutational status

In 1999, a major breakthrough in CLL prognostication was achieved when the mutational status of the *IGHV* genes was found to divide CLL patients into two divergent subgroups following either a favorable or aggressive outcome^{38,39}. Patients carrying *IGHV* genes with $\geq 98\%$ identity to the

corresponding germline gene, were defined as unmutated, while mutated cases were classified as those displaying <98% identity. This cut-off value of 98% was chosen to exclude any potential polymorphic variants^{38,54}. Approximately half of all CLL cases were mutated and had a considerably longer overall survival and time to initial treatment than those with unmutated genes^{39,55}. Today, the *IGHV* mutational status has emerged as one of the strongest prognostic markers in CLL. Furthermore, the fact that the *IGHV* mutational status remains constant throughout the disease course allows precision in predicting the clinical outcome at time of diagnosis, although exceptions do exist such as “borderline” mutated cases and cases with *IGHV3-21* usage⁵⁶⁻⁵⁹.

Genomic aberrations

There is, as yet, no single genetic aberration that has been found in all CLL patients. Nevertheless, several common recurrent genomic aberrations have been identified which have been shown to be of strong prognostic value⁶⁰. In fact, more than 80% of CLL cases carry one or more of the following aberrations; del(13q), trisomy 12, del(11q) and del(17p)⁶⁰.

The most frequent aberration is del(13)(q14.3), which is identified in more than 55% of CLL cases and is associated with an indolent disease course⁶⁰⁻⁶². Both heterozygous and homozygous del(13q) are frequently observed, although homozygous deletions are less common and are observed in about 20% of patients. This deleted region on 13q was found to encode two micro-RNA genes, *miR-15a* and *miR16-1*. These micro-RNAs, are involved in down-regulation of the anti-apoptotic gene *BCL2*, which is often found to be over-expressed in CLL^{63,64}.

The second most common chromosomal abnormality in CLL, detected in 12-18% of all patients, is a deletion of part of the long arm of chromosome 11, and this is associated with poor outcome and lymphadenopathy⁶⁰⁻⁶². The affected region is larger than the deletion on chromosome 13q and spans bands q22 and q23 of chromosome 11^{65,66}. This deletion includes several tumor suppressor genes such as *ATM*, *FDX*, *MLL* and *RDX*^{65,67}. Of these genes, the ataxia telangiectasia mutated (*ATM*) gene is the most promising candidate within this region since it is involved in the regulation of many signaling pathways which respond to DNA damage, and it has also been found to phosphorylate p53, hence triggering the p53-dependent signaling pathways⁶⁸. Moreover, it has been reported that one third of CLL patients with del(11q) carry germline or somatic mutations of the *ATM* gene^{69,70}.

Trisomy 12 which is associated with an intermediate outcome and short time to treatment, has been reported at a frequency of 11-16%^{60,62}. A partial trisomy of 12q13-q15 which contains the oncogenic murine double minute 2 (*MDM2*) gene has been identified in a limited number of cases^{71,72}. The

MDM2 gene is an important feedback regulator of *TP53* and therefore a significant player in cell-cycle control, and has been found to be dysregulated in CLL^{73,74}. A further gene of interest in the context of CLL development is the CLL up-regulated gene 1 (*CLL1*), located at 12q22, which has been shown to be specifically expressed in CLL and not in normal tissue or in other hematological malignancies⁷⁵. However, this gene was not found to be significantly upregulated in CLL cases with trisomy 12.

Finally, deletion of 17p, which encompasses the tumor suppressor gene *TP53*, is found in 5-10% of CLL patients⁶⁰. Considering the crucial role played by *TP53* in maintaining genomic stability, it is perhaps not surprising that deletion of 17p is associated with the worst survival, as well as the shortest time to treatment among patients⁶⁰. In addition, these patients are commonly found to display other poor prognostic markers such as unmutated *IGHV* genes and high expression of CD38 and ZAP70 (described below)⁷⁶. It has also been shown that patients carrying del(17p) do not respond to treatment with purine nucleoside analogues and alkylating agents^{60,62,77-81}. However, an alternative treatment using the monoclonal antibody alemtuzumab has shown promising results in this group of refractory patients^{48,82,83}. It has also been shown that del(17p) and del(11q) patients tend to have greater genomic instability and consequently exhibit higher genomic complexity than patients carrying one or none of the other recurrent aberrations^{61,62}. Furthermore, recent studies have shown that some patients with del(17p) also carry *TP53* mutations on the other allele, conversely, patients may carry the *TP53* mutations without having a deletion of 17p^{84,85}. In addition, it has also been shown that patients carrying either del(17p) or a *TP53* mutation alone have an equally poor prognosis to patients carrying both del(17p) and *TP53*, thus illustrating the importance of *TP53* as an independent prognostic marker⁸⁶. An overview of the common chromosomal aberrations found in CLL are listed in Table 1.

Table 1: Overview of common chromosomal aberrations found in CLL

Known chromosomal aberration	Genes affected	Clinical outcome	Frequency (%)
del(13)(q14)	<i>miR15, miR16</i>	Good	55
del(11)(q23)	<i>ATM</i>	Intermediate/poor	12-18
trisomy 12	-	Intermediate	11-16
del(17)(p13)	<i>TP53</i>	Poor	5-10

Since CLL cells have proved difficult to grow in culture, use of certain growth stimulants such as CpG oligonucleotides, IL-2 and CD40L have allowed better detection of chromosomal aberrations using conventional cytogenetics⁸⁷⁻⁸⁹. This has resulted in an improved detection rate of chromosomal translocations, which were previously thought to be rare events in CLL. Translocations are now reported to be found in as many as

20-30% of patients^{61,90,91}, and both balanced and unbalanced translocations have been associated with poor clinical outcome^{90,92}. In CLL, chromosomal translocations commonly involve the *IGH* locus located at 14q32⁶¹. One of these translocations is t(14;19)(q32;q13) involving both the *IGH* locus and the *BCL3* gene. This translocation is primarily found in patients carrying *IGHV4-39/IGHD6-13/IGHJ5* rearrangements and is associated with poor prognostic markers such as unmutated *IGHV* genes⁹³.

CD38

The transmembrane glycoprotein CD38 is expressed and found on the cell surface of all lymphocytes, including B-cells, and plays a vital role in cell adhesion and calcium flux into the cell⁹⁴. CD38 also plays an important role in BCR signaling and can thereby influence apoptosis in B-cells^{95,96}. In CLL, CD38 expression has been shown to have prognostic value and correlates with disease progression. Employing a 30% threshold value (where >30% of leukemia cells express CD38), high expression of CD38 was shown to be associated with unmutated *IGHV* genes and conversely, low CD38 expression levels were demonstrated to be associated with mutated *IGHV* genes³⁸. However, while some groups reported CD38 expression as a surrogate marker for *IGHV* mutation status, other studies could not verify this^{76,97}. Furthermore, the best cut-off value to define CD38 positivity has been debated and several groups have proposed lower threshold values such as 20%, 7% and 5%^{98,99}. Additionally, it is unclear whether CD38 expression is stable enough over time to be a reliable prognostic marker at early stage disease^{38,100,101}. Nevertheless, CD38 is currently regarded as an independent prognostic marker in CLL, although not considered the strongest¹⁰².

ZAP70

The protein Zeta-chain-associated protein kinase 70 (ZAP70) is an intracellular tyrosine kinase normally expressed by T-cells where it plays an important role in the regulation of T-cell functions; specifically receptor signaling and T-cell activation, cell migration and apoptosis¹⁰³. In a study by Rosenwald *et al*, ZAP70 was found to be highly expressed in *IGHV* unmutated cases following GEP of CLL patients⁴¹. This finding created much interest in the potential of this gene and its protein as a surrogate marker for *IGHV* mutation status and encouragingly, the first study to fully investigate this relationship reported that ZAP70 expression levels predicted *IGHV* mutation status with 93% accuracy^{41,104}. Furthermore, ZAP70 expression appeared to have clinical relevance, in that cases displaying high expression levels showed an unfavorable clinical course in terms of disease progression and overall survival^{105,106}. However, subsequent investigations have highlighted ambiguity between these two prognostic markers with some studies reporting this discordance to be as high as 25%^{107,108}. For example, ZAP70 was observed to be highly expressed in cases with mutated *IGHV3-21* genes while cases with del(11q) and del(17p) displayed low

levels of ZAP70 despite being unmutated¹⁰⁹. Today, ZAP70 expression is considered to be an independent prognostic marker as it can be measured realtime quantitative PCR (RQ-PCR) and flow cytometry and appear to remain stable over time¹⁰⁷.

Stereotypy and the role of antigens in CLL

As mentioned, the approximately 2.3×10^{12} possible combinatorial events occurring during *IGH* and light chain rearrangement as well as the further diversity observed at the junctional regions of the V(D)J rearrangement are essential for the creation of a vast antibody repertoire. Hence, the possibility that two independent B-cell clones carry identical IGs is negligible. Furthermore, the recent finding of preferential *IGHV* gene usage and subsets of CLL displaying remarkably similar BCR features has strongly implicated antigenic recognition in the pathogenesis of CLL.

Biased *IGHV* gene repertoire

It is now well established that CLL displays a remarkably biased *IGHV* gene repertoire with an over-representation of a limited number of genes. Predominating *IGHV* genes, reported by several groups, include *IGHV1-69*, *IGHV4-34*, *IGHV3-7*, *IGHV3-23* and *IGHV3-21*^{36,37,39,56,110}. Recent studies have also reported a skewed usage of *IG* light chain genes¹¹¹. In addition, certain *IG* genes (e.g. *IGHV1-69*) are preferentially observed in unmutated cases, whilst *IGHV4-34* and *IGHV3-7* rearrangements are frequently observed in mutated cases³⁷.

Subsets with stereotyped BCRs

More recently, several groups have reported the existence of multiple CLL subsets with quasi-identical, 'stereotyped' BCRs in up to 30% of patients^{37,57,58,112-116}. These patients carry similar or identical *IG* heavy and light chain genes as well as restricted CDR3 sequences¹¹⁵. In order to define subsets, certain criteria have to be met such as usage of similar *IGHV/IGHD/IGHJ* genes, light chain genes and an amino acid identity of $\geq 60\%$ in the heavy chain CDR3 (HCDR3)^{58,112,114,115}. Considering the aforementioned low probability that two BCRs carry identical CDR3s, these findings have strongly inferred a role for antigen involvement, whereby patients belonging to a subset recognize similar antigen(s)^{114,115,117}. In a recent report, more than 100 different subsets with 'stereotyped' HCDR3 sequences were identified in a series of 1939 CLL patients¹¹⁴. Several of these subsets, for example subset #1, #2 and #4, carry specific combinations of *IG* heavy and light gene rearrangements such as, *IGHV1/5/7/IGKV1-39* (subset #1), *IGHV3-21/IGLV3-21* (subset #2), and *IGHV4-34/IGKV2-30* (subset #4) (Table 2). Interestingly, an association with clinical features has been observed for certain subsets, suggesting that the antigenic binding site may be of importance for determining clinical outcome¹¹⁵.

Subset #1

Subset #1, the most common subset represented at cohort level at a frequency of 2.7%, is characterized by the expression of one of the *IGHV1/5/7* clan genes (e.g. *IGHV1-2*, *IGHV1-3* or *IGHV5a*) together with *IGKV1-39/ID-39* usage¹¹⁵. This subset consists of a HCDR3 sequence of 13-14 codons and carries unmutated *IGHV* genes. Patients belonging to subset #1 have been shown to have a poor prognosis and also express high levels of CD38¹¹⁵. In contrast, non-subset #1 cases using the same *IGHV* genes carry diverse *IG* light chain genes, variable *IGHV* mutational status as well as a heterogeneous CD38 expression and follow a variable clinical course. Interestingly, some subset #1 cases have been shown to bind to oxidized low-density lipoprotein (oxLDL) which is displayed on apoptotic cells¹¹⁸.

Subset #2

The *IGHV3-21* gene is detected in approximately 5% of CLL cases^{114,119}. This group follows an aggressive course with short overall survival time irrespective of *IGHV* mutation status (2/3 of these cases carry mutated *IGHV* genes)¹¹⁹. For patients carrying the *IGHV3-21* gene, approximately half display a stereotyped BCR (defined as subset #2)^{120,121}. This subset shows peculiar molecular characteristics such as a short and homologous HCDR3, typically with 9 codons, but also restricted expression of the *IGLV3-21* gene⁵⁸. Non-subset *IGHV3-21* patients appear to have an equally poor overall survival similar to subset #2 patients^{57,121,122}. That notwithstanding, subset #2 patients both display a shorter time to progression and poor-prognostic markers such as high CD38 expression^{115,123}.

Subset #4 and #16

The *IGHV4-34* gene is detected in approximately 8% of CLL cases and these patients generally display highly mutated *IGHV* sequences, follow an indolent disease course and have a favorable prognosis¹¹⁴. Among *IGHV4-34* expressing CLL cases, four stereotyped subsets have been identified to date (#4, #11, #16, #29)¹¹⁵. The largest of these subsets, subset #4 carries a restricted HCDR3 of 20 amino acids in length and demonstrates preferential *IGKV2-30* usage. This subset is represented at cohort level at a frequency of more than 1% and is mainly comprised of young patients compared to the non-subset #4 patients (median age at diagnosis 43 vs. 63 years), and follows a strikingly indolent disease course¹¹⁵. Furthermore, subset #4 cases have low expression of CD38¹¹⁵. Only a small proportion of CLL cases (~6%) have been reported to be IgG-switched, however subset #4 cases have been found to be uniformly class-switched^{112,115,124}. Interestingly, a report has found an association between persistent infections by Epstein-Barr virus (EBV) and cytomegalovirus (CMV) and subset #4 CLL¹²⁵. In addition, in a recent study, extensive intraclonal diversification within subset #4 cases was observed, further supporting the notion of an active interaction with

antigen(s)¹²⁶. A less frequent subset utilizing the *IGHV4-34* gene is subset #16 (represented at cohort level at a frequency of 0.3%), which is found to have a distinctive HCDR3 of 24 amino acids in length together with restricted *IGKV3-20* gene usage. These cases have also been shown to be IgG-switched, however, little is known regarding clinical outcome for this subset¹¹⁵.

Table 2. Overview of selected subsets in CLL.

Subset	<i>IGHV</i> gene	Predominant <i>IGK/IGL</i> gene	HCDR3 aa length	Clinical outcome
Subset #1	<i>IGHV1/5/7</i>	<i>IGKV1-39/ID-39</i>	13-14	Poor
Subset #2	<i>IGHV3-21</i>	<i>IGLV3-21</i>	9	Poor
Subset #4	<i>IGHV4-34</i>	<i>IGKV2-30</i>	20	Good
Subset #16	<i>IGHV4-34</i>	<i>IGKV3-20</i>	24	Not known

The role of antigens in CLL pathogenesis

As mentioned, the finding of a skewed repertoire of both *IG* heavy and light chain genes and stereotyped CDR3s supports the notion that antigens, either auto-antigens or foreign antigens could stimulate the CLL clone. The theory is that B-cells with certain BCRs are stimulated by antigens, undergo rapid clonal expansion and thereby attain an increased risk of acquiring genetic aberrations and undergoing malignant transformation.

Studies on B-cell reactivity in CLL have revealed that cases with unmutated BCRs display both autoreactivity and polyreactivity towards molecules such as DNA, insulin and lipoprotein lipase (LPL)^{127,128}. In contrast, *IGHV* mutated cases did not display these properties^{128,129}. Recent studies have also found that CLL IGs can specifically bind to certain antigens such as cytoskeletal proteins, e.g. vimentin, filamin B and cofilin-1 expressed on apoptotic cells^{117,118}. In addition, it was also observed that phosphorylcholine-containing antigens, e.g. *Streptococcus pneumoniae* polysaccharides and oxLDL, could also specifically bind to CLL IGs¹¹⁸. As mentioned, persistent infections by EBV and CMV have been reported in subset #4 CLL cases¹²⁵. These recent findings have indicated that the CLL clone could possibly arise from B-cells that have similar function to scavenger cells and hence recognize apoptotic remains but also have the ability to bind conserved bacterial cell structures¹³⁰.

IGHV3-21, and in particular stereotyped subset #2 patients have demonstrated a tendency to maintain the germline configuration in the binding motif recognized by Staphylococcal protein A, leaving open a potential role for superantigenic involvement in the pathogenesis of CLL¹¹⁴. More specifically, the framework regions (FR1, FR3) and the HCDR2 of the *IGHV3* subgroup have been found to interact with certain bacterial

superantigens^{127,131,132}. Thus, it should be kept in mind that antigenic stimulation of CLL tumors may occur outside of the typical IG antigen binding site, i.e. the HCDRs. Furthermore, subset #2 antibodies have been shown to bind to the auto-antigen cofilin-1, an actin-binding protein found to co-localize with molecular complexes on the cell surface during apoptosis¹¹⁸.

IGHV4-34 is known to encode antibodies that bind to self antigens and has been observed to be inherently auto-reactive in its germline state, through the recognition of the N-acetyllactosamine (NAL) antigenic determinant of the I/i blood group antigen^{133,134}. Moreover, anti-I/i *IGHV4-34* antibodies also bind the poly-NAL in the B-cell isoform of CD45^{135,136}. These findings may explain why the *IGHV4-34* antibodies binds to viable B-cells¹³⁷. This I/i antigen may be expressed on oxidized apoptotic cells and CD45 is expressed by pre-apoptotic T-cells. Furthermore, *IGHV4-34* antibodies demonstrate cross-reactivity with several auto- and exo-antigens, and based on this occurrence, it is possible that *IGHV4-34* genes must undergo SHM in order to negate their autoreactivity and subsequently become more “safe” in order to remain within the functioning IG repertoire.

Mantle cell lymphoma

Introduction

Mantle cell lymphoma (MCL) is considered to be one of the most aggressive lymphoid neoplasms and has a median survival of only 3-5 years¹³⁸. MCL is called a ‘mantle’ cell lymphoma because it first arises within a region of the lymph node called the ‘mantle’ zone. This disease predominantly affects elderly males (male:female ratio, 3:1) with a median age at diagnosis of 65 years^{139,140}. Approximately 70 patients are diagnosed with MCL every year in Sweden.

At diagnosis, the disease in most patients involves not only lymph nodes but also other sites of the body. These sites may include the spleen, bone marrow and blood, the lymph nodes in the throat (tonsils and adenoids), the liver, and/or the gastrointestinal tract¹⁴¹. Symptoms experienced by patients with MCL include loss of appetite, weight loss, nausea, indigestion, abdominal pain or discomfort due to an enlarged liver or spleen.

The hallmark of MCL is the over-expression of the cyclin D1 protein, usually as a consequence of the t(11;14)(q13;q32). In addition, MCL cells usually express CD5, CD19, CD20, CD22, CD79a, and have a strong surface IgM expression. In MCL γ light-chain restriction predominates over κ restriction, which is unusual among B-cell lymphomas^{1,142,143}. Both MCL

and CLL express CD5, however MCL is usually CD23 negative and over-expresses cyclin D1 whereas surface IgM is considerably weaker in CLL. The cause of this incurable malignant disease is unknown. The clinical course is usually aggressive, although recently several groups have identified a subset of MCL patients that displays an indolent clinical course¹⁴⁴⁻¹⁴⁸.

IGHV genes

In comparison to CLL, MCL patients often carry unmutated *IGHV* genes, although a minority of MCL patients has been found to carry mutated *IGHV* genes^{149,150}. However, the mutational load is lower in comparison to *IGHV* mutated CLL patients. Currently, MCL is believed to originate from mature naive B-cells. However, restricted usage of *IGHV* genes has also been reported in MCL with an overrepresentation of *IGHV3-21*, *IGHV4-34* and *IGHV5-51* genes¹⁴⁹. Moreover, it has also been shown that unlike CLL where patients expressing *IGHV3-21* genes have a poor prognosis, *IGHV3-21* MCL patients have better prognosis and a lower number of genomic alterations^{150,151}.

Gene expression profiling in MCL

In 2003, a distinct gene expression signature was reported for MCL by Rosenwald *et al*¹⁵², and this profile differed from that of other lymphomas such as CLL and both the GC B-cell-like and activated B-cell like type of DLBCL. This gene expression profile also revealed 20 genes associated with proliferation which could identify a subset of patients with a shorter survival¹⁵². Recently, a comparative genomic and GEP study in MCL identified a subset of patients displaying a lack of genomic aberrations and hence having a better prognosis¹⁵³.

Genetic alterations

The translocation t(11;14)(q13;q32) is regarded as a primary event occurring in MCL cells and is present in almost all MCL cases¹⁵⁴. This translocation can be detected using fluorescence *in situ* hybridization (FISH). It involves the placement of the cell cycle regulatory protein cyclin D1 (*CCND1*) within close proximity of the *IGH* locus¹⁵⁵⁻¹⁵⁷. Consequently, the *CCND1* gene (also known as *PRAD-1* or *BCL1*) located on chromosome 11, will be juxtaposed to the active enhancer of the *IGH* locus resulting in an over-expression of the cyclin D1 protein. Another genetic abnormality commonly observed and found in 20-40% of MCL patients, is the 11q22-23 deletion involving the *ATM* gene¹⁵⁸⁻¹⁶¹. Additionally, it has been shown that the even though the *ATM* gene is not deleted it can be inactivated by mutations, but also affected by both these combinations (deletion and mutation)¹⁶². The *ATM* gene plays

an important role in response to double strand DNA breaks and phosphorylates p53 in the presence of DNA damage. Hence, loss of the *ATM* gene increases the risk of other chromosomal aberrations. Furthermore, it has been demonstrated that the t(11;14) is insufficient for tumorigenesis in mouse models and is therefore thought to be a primary event in MCL, contributing to genetic instability¹⁶³. Additional secondary alterations reported in MCL include losses at 1p13-p31, 6q23-q27, 9p21 and 9q, 11q22-q23, 13q11-q13, 13q14-q34, 17p13 and 22q but also gains at 3q26, 7p21, 8q24¹⁶⁴⁻¹⁶⁷.

Treatment

Today, no curative treatment is available for MCL and long term remission is very rare; however, survival rates have improved as new treatment alternatives have emerged^{168,169}. The introduction of high dose chemotherapy and addition of anti-CD20 monoclonal antibody (rituximab) have significantly improved the prognosis¹⁷⁰. Furthermore, the outcome for elderly patients has improved with the use of multiple-drug combinations and rituximab¹⁷¹. Currently, the only curative treatment for MCL is allogeneic bone marrow transplantation, nevertheless, transplantation related complications unfortunately render this option unsuitable for the majority of patients¹³⁸.

Array based technologies

Genomic arrays

Array-based comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays are two high resolution techniques that measure copy-number alterations (CNAs) and thus are important tools for studying genetic events in for instance cancer and developmental disorders.

CGH is a quantitative method based on the comparative hybridization of two samples (patient/tumor and a reference sample), labeled with different fluorescent colors, to metaphase spreads from a healthy control¹⁷². Large scale genomic alterations can then be detected based on the fluorescence ratio of the hybridized DNA samples from the patient compared to the reference. CGH-arrays are a further development of the same principle. Here, hybridization of labeled DNA is performed on microarray slides that contain probes, each representing a unique DNA sequence. This method allows a higher resolution and provides the exact positions of the chromosomal aberration compared to conventional CGH.

SNP-arrays are at present predominantly used in research for genotyping and screening of genomic aberrations, yet are also applied for diagnostic and prognostic purposes. SNP-arrays offer high resolution power and can detect small changes in copy number (~10-25kb) but can also detect copy neutral loss of heterozygosity (CNN-LOH). For the work presented within this thesis, we have applied Affymetrix SNP-arrays for whole-genome screening which is described below.

Single nucleotide polymorphism arrays

The Affymetrix GeneChip SNP-array consists of a square glass substrate mounted in a plastic cartridge where the glass contains an array of oligonucleotides each 25bps in length. For each SNP, different oligonucleotides of 25bp are tiled, all with a slight variation in perfect matches, mismatches, and flanking sequences surrounding the SNP. Tumor DNA is fragmented using the restriction enzyme *Nsp1*, and adapters are then added to allow PCR amplification of the fragments. Following the amplification, fragments are again fragmented and labelled. These labeled fragments are then hybridized to the microarray chip, the non-bound material is washed off, and the signals emitted from the fluorescent probes are detected, measured and stored for analysis (www.affymetrix.com).

Affymetrix currently manufactures SNP-array chips incorporating 10K to 2.7M markers, thereby providing great resolution to enable the detection of both known and novel aberrations throughout the entire genome. Consequently, SNP-array technology allows the alignment of SNPs in chromosomal order and the identification of chromosomal alterations such as CNAs and CNN-LOH (explained below) (Figure 2). In a recent CLL study, a 250K Affymetrix *Nsp* array was subsequently validated for routine clinical use¹⁷³ and was demonstrated to have 98.5% concordance with the standard CLL FISH panel.

Copy Neutral Loss of Heterozygosity

CNN-LOH, also known as uniparental disomy (UPD), is observed in both hematological and solid tumors^{174,175}. In CNN-LOH, one allele is duplicated whilst there is loss of the other allele, hence a genomic event has taken place without a change in copy-number (Figure 2). This may lead to clinical conditions by producing either homozygosity for recessive mutations or else aberrant patterns of imprinting¹⁷⁵. Through the use of SNP-arrays, CNN-LOH has been found to be a common event in many types of cancers and as such serves as an alternative to a deletion, in terms of the 'second hit' in the Knudson two hit hypothesis of tumorigenesis^{176,177}. The advantage of using SNP-arrays is that they can detect both copy number, LOH (i.e. a deletion) and CNN-LOH in comparison to FISH and array-CGH which can only detect copy-loss LOH¹⁷³.




Copy number aberration	Copy-number change
Copy number loss	Loss of genomic DNA 
Copy number gain	Gain of genomic DNA 
Copy number neutral loss of heterozygosity	Allelic imbalance without a change in DNA copy number 

Figure 2: Genomic aberrations, CNA and CNN-LOH.

Recent SNP-array findings in CLL and MCL

In CLL, FISH analysis is the current gold standard for detection of recurrent genomic aberrations. However, a drawback of FISH is its inability to detect novel aberrations. In recent years, high-resolution array-based platforms, including SNP-arrays have made possible the identification of novel deletions or amplifications in CLL including gain of 2p and 8q and deletion of 4p, 8p and 22q^{62,178-180}. However, these aberrations are found at varying frequencies and their clinical significance remains unclear. In CLL, CNN-LOH covering large parts on chromosome 13q has been reported in patients with homozygous del(13q), and may thereby introduce an alternative disease mechanism to the loss of the miRs in this region^{62,181}.

In a recent publication using high-resolution SNP-arrays in CLL, it was noted that there was an association between increasing genomic complexity and survival. Specifically, patients with two or more genomic aberrations displayed significantly poorer clinical outcome¹⁸². Furthermore, it has been reported that patients carrying del(17p) and del(11q) harbor larger genomic aberrations at a considerably higher frequency than patients with other recurrent aberrations⁶². In a study performed on 203 newly diagnosed patients with CLL, it was noted that 50% of patients with ≥ 4 aberrations larger than 5 Mbp carried the recurrent aberration del(17p) and the remaining cases all displayed deletions of 11q⁶².

Also, 80% of cases displaying 2-3 CNAs also carried del(11q), del(17p) or trisomy 12. Conversely, CLL patients displaying no recurrent alterations or the good prognostic del(13q) carried no more than 1 CNA larger than 5 Mbp. Furthermore, this study confirmed that a higher number of larger aberrations were associated with worse prognosis. These findings clearly highlight the association between genomic complexity and the nature of the disease, with aggressive cases exhibiting a marked increase in genomic complexity compared to indolent cases.

In MCL, SNP-array studies have revealed a large number of secondary alterations, as mentioned above^{166,183}. Secondary genomic aberrations have been linked to losses at 8p, 9p, 9q, 13q14, and 17p as well as gains at 3q, 8q, and 12q^{164,184,185}. More recently, it was shown that losses at 1p, 1q, 2q, 9p, and gains at 12q was associated with an inferior survival in MCL¹⁶⁶. Furthermore, a correlation between an increased genomic complexity and losses at 8p, 11q, 13q and 17p has recently been described^{166,186}. In addition, with regards to the proliferation signature, deletions occurring at 7p and 9q have been associated with high proliferation scores in MCL^{166,186}.

Gene expression profiling

GEP has become a powerful tool for exploring the molecular biology both within and between heterogeneous entities and enables the identification of genes or classes of genes, which may serve as biomarkers for diagnostics, prognostics, and/or drug discovery purposes. Global GEP array analysis enables us to simultaneously screen thousands of genes in a sample. Today, multiple commercial expression microarrays are available and one of the most renown within this field is the Affymetrix GeneChip.

For a typical gene expression analysis, RNA is isolated, labeled and then hybridized to a GeneChip for example, Affymetrix Human Genome U133 Plus 2.0 GeneChip® microarray, which contains millions of copies of DNA fragments arranged on a glass slide (www.affymetrix.com). Non-bound material is washed off and the signals emitted from the fluorescent probes are detected, measured and stored. The signals are then normalized and the values from the corresponding genes can be analyzed using various software programs (Figure 3).

A filtering is performed prior to analysis to exclude genes that do not vary between samples and are therefore of low biological relevance for the study. Following this, genes of interest can be clustered by either supervised or unsupervised hierarchical clustering. In an unsupervised hierarchical clustering, samples and genes are clustered together according to similarities in gene expression. Supervised hierarchical clustering is based on creating groupings of specific variables such as genes or samples.

Gene expression profiling in hematological malignancies

In 1999, Golub *et al* performed the first microarray analysis with an aim to identify differences between acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL)¹⁸⁷. They demonstrated the usefulness of GEP for both the classification of cancers as well as the prediction of unknown patient samples. Several hematological malignancies have since been investigated using GEP such as diffuse large B-cell lymphoma (DLBCL), MCL and multiple myeloma (MM)^{152,188-191}. GEP performed on these malignancies has been essential in order to explore prognostic markers, new subgroups, drug responses as well as understanding the pathology behind these diseases.

In CLL, several GEP array studies have been performed in order to gather knowledge and hence, further understand the biology behind this malignancy. As mentioned, Rosenwald *et al* and Klein *et al* divided CLL patients into two subgroups based on their *IGHV* mutational status in order to investigate the GEP within these groups^{40,41}. To that end, unsupervised clustering was performed on both unmutated and mutated CLL, where the results demonstrated that CLL cases displayed a common GEP independent of their *IGHV* mutational status^{40,41}. However, a set of genes was found to discriminate between mutated and unmutated genes, and one such gene was the aforementioned *ZAP70* gene⁴¹. Furthermore, Rosenwald *et al* also showed that CLL displayed a specific signature distinct from other lymphoid malignancies and normal B-cell subpopulations. Moreover, comparison of CLL profiles to those of purified B-cells from healthy individuals indicated that the common CLL profile resembles that of a memory B-cell rather than a naive B-cells⁴⁰. This finding supported the idea that all CLL, regardless of the level of SHM present in the *IGHV* gene, had encountered antigens.

In 2005, Fält *et al* studied GEP in CLL patients carrying the *IGHV3-21* gene¹⁹². They reported a distinct expression profile for *IGHV3-21* patients in comparison to non-*IGHV3-21* mutated and unmutated patients. Furthermore, a set of genes was identified that could distinguish *IGHV3-21* patients from non-*IGHV3-21* patients. Such genes included those involved in regulation of DNA replication/cell cycle control, transcription and protein kinase activity. The deregulation of these genes expressed in *IGHV3-21* patients was suggested to reflect their clinically poorer outcome¹⁹².

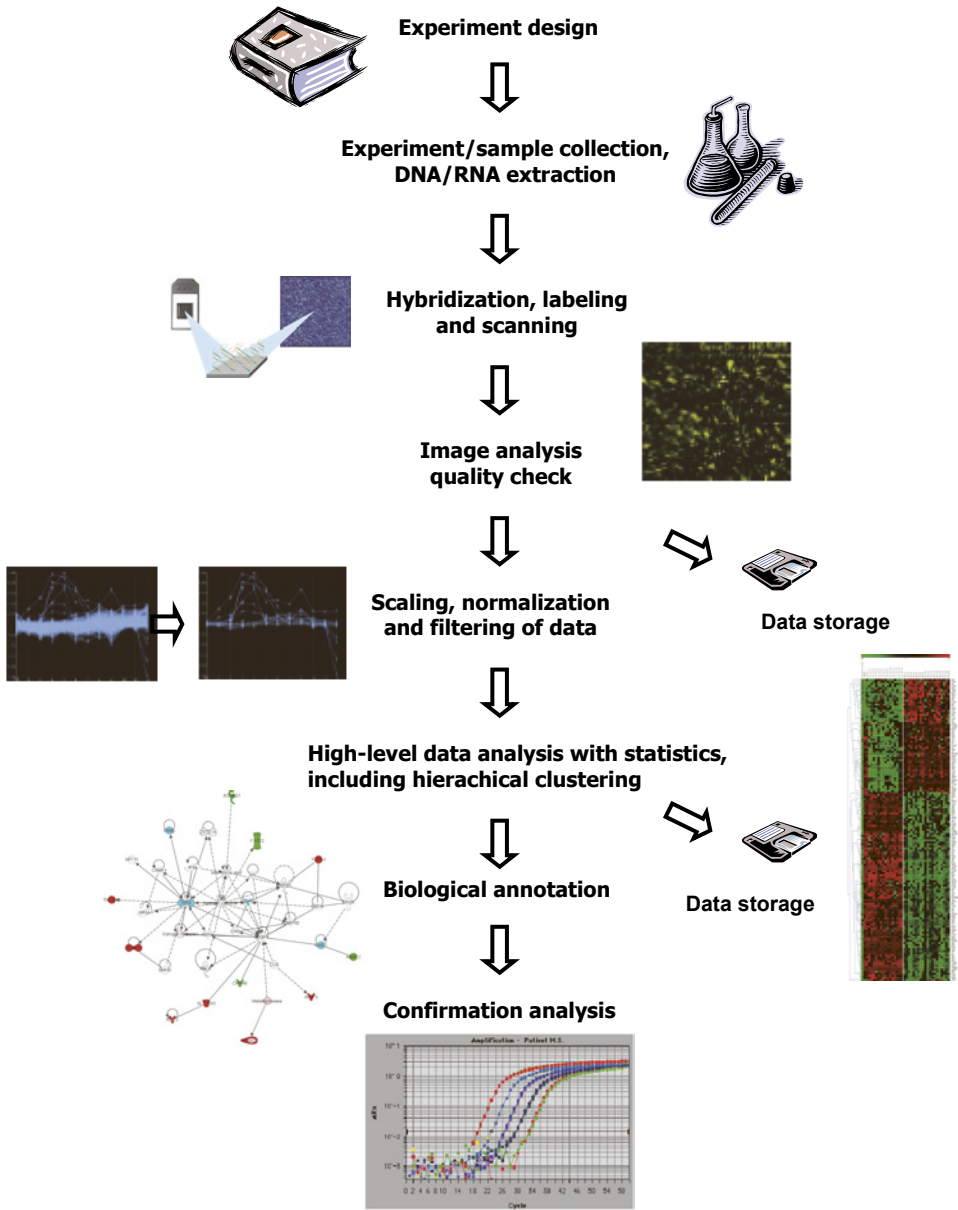


Figure 3: Brief outline of the workflow of a general array design.

Epigenetic changes by DNA methylation

DNA methylation is normally involved in tissue specific gene expression, genomic imprinting, embryogenesis, and observed in some tissues as a function of aging^{193,194}. The process of methylation occurs at CpG dinucleotides where methyltransferase enzymes catalyze the addition of a

methyl group to the number 5 carbon of a cytosine that is immediately 5' to a guanine (CG dinucleotide). CpG islands are regions of DNA that have a higher percentage of CG dinucleotides (>60%) than the rest of the genome, and usually range in size from 200bp to 5kb. These CpG islands are distributed throughout the genome and can be located in exons, introns and promoters^{195,196}.

Hypermethylation is a common term used in the field of epigenetics, and signifies an increase in the methylation of cytosine residues. Conversely, hypomethylation refers to a decrease in methylation at cytosine residues throughout the genome. The epigenetically mediated silencing from hypermethylation results from blockage of transcription recognition by CpGs sites thereby inhibiting transcription¹⁹⁷.

Aberrant methylation can play an important role in tumorigenesis since genes involved in tumor suppression, growth regulation, differentiation, or apoptosis have all been shown to be adversely affected by this epigenetic mechanism¹⁹⁸⁻²⁰⁰. A strong association between DNA promoter methylation and transcriptional inactivation has been reported with the end result being functionally similar to a genomic deletion or inactivating mutation¹⁹⁷. Consequently, it is evident that abnormal DNA methylation plays an important role in tumor progression since it encompasses genome-wide hypomethylation and promoter CpG island hypermethylation²⁰¹. However, DNA methylation is a potentially reversible modification and de-methylating chemotherapies are currently being developed and considered for cancer treatment^{202,203}. Moreover, the loss of DNA methylation can lead to mitotic recombination, chromosomal instability and activation/inactivation of proliferative genes²⁰⁰.

Histone modifications have been shown to play a key role in epigenetic regulation²⁰⁴. Alteration of chromatin is reliant on histone modifications and it is known that histones can be subjected to more than 100 different modifications including; acetylation, methylation, phosphorylation and ubiquitination^{205,206}. Today, several studies have emerged indicating that in addition to DNA methylation, the epigenetic regulation of histone modification may play a fundamental role in the development of cancer^{206,207}.

Methylation arrays

One of the first methods employed for the analysis of DNA methylation was by using sensitive restriction enzymes, coupled with Southern blot analysis of DNA fragments²⁰⁸. This technique distinguishes methylated from unmethylated sites but was rather time consuming and incomplete digestion with restriction enzymes was a problem. Another technique, high performance liquid chromatography, focuses on the quantification of the

total amount of methylation but lacks the ability to provide information about the actual sequence²⁰⁹. The application of bisulfite conversion techniques in conjunction with PCR amplification and bisulfite/pyrosequencing was a major breakthrough in the study of epigenetics. Bisulfite treatment deaminates cytosine to uracil but leaves the 5-methylcytosine unaffected^{210,211}. If bisulfite treated, DNA is amplified by PCR, 5-methylcytosine on the template strand pairs with guanine on the newly synthesized strand but cytosine, which is now converted to uracil, pairs with adenine. Hence, if a cytosine is unmethylated, it is occupied by thymine, however, if it is occupied by cytosine this is interpreted as having been methylated, as illustrated in Figure 4. Following this, bisulfite/pyrosequencing methods can be applied to determine the bisulfite-converted sequence of the specific CpG sites.

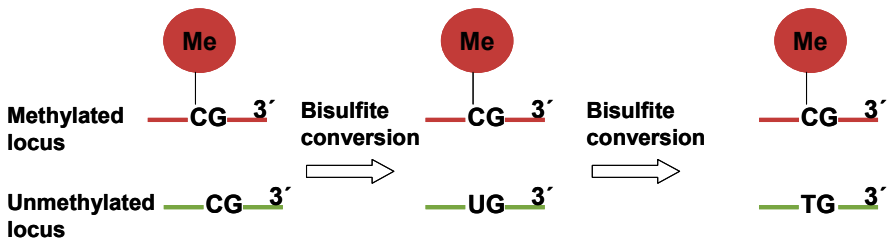


Figure 4: Bisulfite conversion of genomic DNA.

Designing a microarray to investigate DNA methylation at a genome-wide level is a challenging problem since issues regarding both the resolution (coverage) of probes and/or where such probes should be located must be considered. A popular choice of method involves bisulfite conversion of genomic DNA in combination with arrays that distinguish between converted unmethylated CpG loci and unconverted methylated CpG loci.

The Illumina Methylation Assay incorporating the Infinium II platform uses the "BeadChip" technology in order to generate a comprehensive genome wide profiling of human DNA methylation²¹². This method quantifies the methylation levels at specific loci throughout the genome. Although the entire human genome is not covered by the array, this technology can measure methylation at 27,578 CpG dinucleotides spread across 14,495 genes.

In brief, bisulfite converted DNA is whole-genome amplified, the products are then enzymatically fragmented, precipitated, resuspended and finally hybridized overnight to locus-specific oligonucleotide primers on the BeadArray. Following hybridization, the cytosine or thymine nucleotides are detected by single-base primer extension and fluorescence signals corresponding to either of these nucleotides are then measured from the

BeadArrays using an Illumina BeadStation GX scanner²¹³. Thereafter, the fluorescence data is analyzed using software which normalizes the raw data, thereby reducing the effects of experimental variation and background. In addition, standard statistical tests can thereafter be applied on the data set.

Methylation studies in hematological malignancies

DNA hypermethylation is a common process by which tumor suppressor genes become inactivated in both leukemias and lymphomas^{199,214,215}. In B-cell lymphomas, several genes have been shown to be affected by hypermethylation silencing such as *ZAP70*, *DAPK1*, *p57* and *p16INK4a*²¹⁶⁻²¹⁹. For instance, Corcoran *et al* investigated the methylation status of the prognostic marker *ZAP70* in CLL. Their analysis revealed a close association between the methylation status and expression of *ZAP70* in CLL cases²¹⁶. Another interesting finding was the down-regulation of death-associated protein kinase 1 (*DAPK1*) expression as a result of methylation in CLL²¹⁷. This gene participates in a wide range of apoptotic signals and is a positive mediator of gamma-interferon induced programmed cell death. Studies in CLL have also shown a strong correlation between promoter methylation and transcriptional silencing for individual genes including *TWIST2* and *HOXA4*^{220,221}. Furthermore, the methylation of several of these genes has been reported to correlate with the *IGHV* mutational status^{222,223}.

In a recent array-based study of hematological malignancies, it was reported that DNA hypermethylation in mature B-cell lymphomas was accompanied by gene-specific hypomethylation which is in contrast to the global hypomethylation described previously²²⁴. Moreover, the heterogeneous methylation patterns observed among B-cell lymphomas may reflect the different cellular origin of each disease²²⁴.

In 2004, Rush *et al* performed the first genome-wide methylation analysis on CLL samples covering ~3,000 CpG sites where they investigated the overall CpG island methylation relative to normal B-cells. To perform this study, they used a technique called Restriction Landmark Genomic Scanning together with bisulfite sequencing. They successfully identified a panel of 195 genes that were abnormally methylated in CLL. Furthermore, they reported that 2-8.1% of the CpG islands were aberrantly methylated and that methylation events showed a non-random distribution in CLL²⁰¹.

Using the Illumina 27K methylation array, our group recently demonstrated significant differences in the methylation patterns between *IGHV* mutated and unmutated CLL subgroups²²⁵. In particular, within the *IGHV* unmutated group, seven methylated tumor suppressor genes as well as eight unmethylated genes enhancing cell proliferation and tumor progression were identified. Of particular interest was the observation that aberrant methylation was associated with inverse changes at the mRNA level. In fact,

reexpression of three candidate tumor suppressor genes has previously been performed (e.g. *IGSF4*, *ABI3* and *VHL*)²²⁵.

More recently, a genome-wide methylation study identified significantly different promoter methylation patterns of MCL patients in comparison to normal naïve B-cells²²⁶. This study by Leschenko *et al* utilized the HpaII tiny fragment Enrichment by Ligation mediated PCR assay and identified a number of genes that function as tumor suppressors to be hypermethylated (e.g. *CDKN2B*, *MLF-1*, *PCDH8* and *HOXD8*), as well as several hypomethylated genes (i.e. *CD37*, *HDAC1*, *NOTCH1* and *CDK5*).

AIMS

The aims of the studies within this thesis were to characterize recently described CLL subsets with stereotyped BCRs regarding patterns of genetic and epigenetic alterations and gene expression profiles. Specifically the aims were as follows;

- I** To apply high-resolution SNP-arrays to screen for genomic aberrations in CLL patients with stereotyped and non-stereotyped *IGHV3-21* and *IGHV4-34* gene rearrangements.
- II** To apply gene expression arrays to characterize the global gene expression profiles in stereotyped and non-stereotyped *IGHV4-34* CLL patients.
- III** To perform array-based methylation profiling in stereotyped subset #1, subset #2 and subset #4 CLL patients.
- IV** To investigate methylation patterns in stereotyped CLL subset #1 and subset #4 in comparison to MCL.

PATIENT MATERIAL AND METHODS

Patient tumor samples

In paper I, 101 tumor samples were collected from participating research institutes in France (n=15), Greece (n=25), Denmark (n=4) and Sweden (n=57). The majority of samples were derived from peripheral blood (n=88), but samples were also collected from bone marrow (n=8) and spleen (n=5). In this study, stereotyped subsets were included and these subsets were defined by certain criteria i) similar usage of *IGHV-D-J* genes and light-chain genes and ii) an amino acid identity of 60% or more in the HCDR3^{114,115}. In total, 42 *IGHV3-21* samples (*IGHV3-21/IGLV3-21*, subset #2, n=29 and non-subset #2, n=13) and 59 *IGHV4-34* samples (*IGHV4-34/IGKV2-30* subset #4, n=17, *IGHV4-34/IGKV3-20* subset #16, n=8, and non-subset #4/16, n=34) were investigated.

In paper II, 25 *IGHV4-34* tumor samples including subset #4 (*IGHV4-34/IGKV2-30*, n=11), subset #16 (*IGHV4-34/IGKV3-20*, n=5), and 9 non-subset samples were collected from collaborating institutes in France (n=3), Greece (n=10) and Sweden (n=12). All samples were derived from peripheral blood. Additionally, all samples were enriched for CLL cells through negative depletion of non-tumor cells (described below).

In paper III, 39 CLL patient samples were included, all derived from peripheral blood, except for two obtained from bone marrow, and collected from collaborating institutes in Greece (n=18) and Sweden (n=21). Here we investigated three major subsets, i.e. subsets #1 (*IGHV1/5/7/IGKV1-39/ID-39*, n=15) and #2 (*IGHV3-21/IGLV3-21*, n=9) and subset #4 (*IGHV4-34/IGKV2-30*, n=15).

In paper IV, a total of 30 CLL samples, including subsets #1 (*IGHV1/5/7/IGKV1-39/ID-39*, n=15) and subset #4 (*IGHV4-34/IGKV2-30*, n=15), were investigated. CLL samples were collected from (Greece, n=18) and Sweden, n=12). All samples were derived from peripheral blood except for two which were obtained from bone marrow. A total of 20 MCL samples derived from lymph nodes were collected from Karolinska University Hospital, Sweden.

CLL patient samples were classified according to the recently revised criteria and displayed the classical CLL immunophenotype and $\geq 70\%$ tumor cells²². All MCL cases fulfilled WHO diagnostic criteria, including over-expression of cyclin D1 (determined by immunostaining) or the presence of t(11:14) determined by FISH¹.

Detailed clinical and molecular data for CLL patients are available in paper I, II III and for MCL in paper IV. In all papers, the *IGHV* mutational status was assessed in all CLL patients, where cases with $\geq 98\%$ identity to germline were classified as unmutated, whereas cases with $< 98\%$ identity were considered mutated.

SNP-array

In paper I, Affymetrix 250K *NspI* SNP-arrays were applied to 101 CLL samples and experiments were performed according to the standard protocols for Affymetrix GeneChip® Mapping 250K arrays. The quality control, genotype calling and probe level normalization was performed by Affymetrix GeneChip® Genotyping Analysis Software (GTYPE) 4.1. As a reference set, 82 normal samples analyzed at the Uppsala Array Platform were used.

Copy number analysis was performed by Nexus Copy Number 3.0 Software (BioDiscovery, El Segundo, CA, USA) employing the Rank Segmentation algorithm. Furthermore, the following thresholds were employed in the copy number analysis: a significance threshold (p-value) of 1×10^{-6} and a \log_2 ratio cut-off at ± 0.2 for regions sized 200-500 kbp and ± 0.15 for regions > 500 kbp, respectively. Moreover, CNAs smaller than 200kbp were not included in the study. These settings were defined based on previous validation experiments on a sample-set including 203 CLL samples⁶². Furthermore, overlapping CNAs with regions of reported CNVs were identified and removed from further analysis as previously described⁶².

Data analysis of CNN-LOH in CLL cells was performed taking into account the fraction of normal cells obtained from flow-cytometry²²⁷. Regions containing CNN-LOH larger than 3 Mbp with less than 50% overlap to CNVs were considered for further analysis.

Cell sorting and RNA preparation

There are different kinds of cell sorting methods, for instance, positive cell selection, in which the cells of interest are sorted out from a sample by binding to a selecting molecule or negative selection, where cells that are not of significance are removed leaving the cells of interest in the sample. These two methods can be employed using small magnetic beads coated with

antibodies that bind to the cells of interest. In paper II and III, negative selection using the Dynal B-Cell Negative Isolations Kit (Invitrogen AB, Sweden) was employed in order to obtain untouched B-cells. The tumor content following purification was verified by fluorescence activated cell sorting (FACS) analysis using antibodies against CD5 and CD19.

Good quality RNA was essential for the overall success of the analysis, both in terms of the array analysis and the verification steps. RNA extraction from the isolated CLL cells was performed using the RNeasy Mini kit (Qiagen Hilden, Germany). The RNA concentration was measured with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA).

Gene expression profiling array

In paper II, the Affymetrix Human Genome U133 Plus 2.0 array (covering over 47,000 transcripts) was used to study the gene expression profile of 25 sorted *IGHV4-34* expressing CLL samples. Biotinylated fragmented complementary RNA (cRNA) was prepared from 100ng of patient RNA using a two-cycle amplification step, according to the GeneChip® Expression Analysis Technical Manual (Rev. 5, Affymetrix Inc., Santa Clara, CA). cRNA was hybridized to the expression arrays for 16 hours in a 45°C incubator with rotation at 60 rpm. The arrays were washed using the Fluidics Station 450 and scanned using the GeneChip® Scanner 3000 7G.

Analysis of the gene expression data was carried out in R (<http://www.r-project.org>) using packages available from the Bioconductor project (www.bioconductor.org). Data was background adjusted and normalized. Differentially expressed genes in subset #4, subset #16 and non-subset samples were analyzed by employing empirical Bayes moderated t-test using the ‘limma’ package. To address potential problems with multiple testing, the p-values were adjusted using the method of Benjamini and Hochberg²²⁸.

Methylation array

In paper III and IV, the genome-wide Illumina Infinium HumanMethylation27 Bead Chip array (Illumina, San Diego, USA), spanning 27,578 CpG sites and covering 14,495 genes was used to analyze the methylation patterns within specific CLL subsets and MCL. This assay is based on the conversion of unmethylated cytosine into uracil (thymine) by bisulfite treatment. Bisulfite converted DNA was subsequently whole-genome amplified, enzymatically fragmented, precipitated, resuspended and hybridized overnight to locus-specific oligonucleotide primers on the BeadArray (Illumina). Fluorescence signals corresponding to either cytosine

or thymine nucleotides were then measured from the BeadArrays using an Illumina BeadStation GX scanner. The intensities of methylated and unmethylated bead types are analyzed and measured whereby the DNA methylation values for each locus, described as Beta values or methylation index (MI), are recorded by BeadStudio software (Illumina). The methylation status can range from 0.1 (completely unmethylated) to 1 (completely methylated). Furthermore, using MI cut-offs of 0.7 or more and 0.4 or less (average of all MI values in each group) were considered as methylated and unmethylated, respectively. Methylation array results were visualized on heatmaps and dendrograms using Genesis TreeView version 1.2.7 (www.genome.tugraz.at).

Gene annotation

Target genes identified through global analyses of patients, for instance by applying array technology, need to be annotated in order to get a comprehensive view of the network of genes affected. By annotating genes of interest, useful information regarding biological function, interaction with other genes and also the pathways that the particular genes/genes are involved in can be obtained. This type of analysis was performed in papers II, III and IV of this thesis.

Gene Ontology Tree Machine (GOTM) is a web-based tool for functional enrichment analysis in various biological contexts (<http://bioinfo.vanderbilt.edu/gotm>). GOTM compares an uploaded gene list to genes in pre-defined functional categories in order to identify those categories containing an enriched number of genes. Statistical analysis is also performed to identify enriched categories. Categories such as cellular components, biological processes and molecular functions provide information and organization of genes of interest. In addition, GOTM provides the number of reference genes within the category (C), the number of genes within the gene set and in the category (O), the expected number of genes in the category (E), the ratio of enrichment (R), the p value from hypergeometric test (rawP), and the p value adjusted by multiple test adjustment.

Ingenuity Pathway Analysis (IPA) is a software program that aids the researcher in understanding the complex biological pathways when analyzing data sets obtained from global analysis platforms including arrays. IPA also provides modeled relationships between proteins, genes, complexes, cells, tissues, drugs, pathways, and diseases (Ingenuity® Systems, Mountain view, CA, USA www.ingenuity.com).

Real-time quantitative polymerase chain reaction

This PCR-based technique can measure DNA copy numbers and expression levels²²⁹. The two most commonly used methods of performing RQ-PCR are probe-based (TaqMan) and intercalated-based (SYBR-Green). SYBR-Green contains intercalating dyes which bind to the synthesized DNA and subsequently emit fluorescence²²⁹. The fluorescent signal in each PCR reaction is then captured at frequent intervals with a highly sensitive camera. RQ-PCR using SYBR-Green master mix (Fermentas, Burlington, Canada) was performed in papers II, III and IV in order to validate array data and measure the gene expression of selected genes. Primers for RQ-PCR validation were designed using Primer3 software (Broad institute, Boston, USA) and PCR reactions were run on the Stratagene Mx 3005p instrument (Stratagene, La Jolla, CA, USA). Furthermore, calculations were performed with Max Pro QPCR software (Stratagene) and using the *Beta-Actin* gene as a reference.

Statistical analysis

Chi-square, ANOVA tests and t-tests were applied to determine any statistical differences between the groups studied (subsets/non-subsets). All statistical analyses were performed using Statistica Software 8.0 (Stat Soft inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Paper I

Genomic screening of stereotyped CLL subsets

Today, more than 100 stereotyped CLL subsets have been identified carrying virtually identical CDR3s¹¹⁴, strongly supporting the role of antigens in disease pathogenesis. Among cases expressing the *IGHV4-34* gene, the main subset - subset #4, displays a restricted HCDR3 of 20 amino acids and *IGKV2-30* usage. A second smaller subset, subset #16, displays a restricted HCDR3 of 24 amino acids in length and *IGKV3-20* usage. Furthermore, subset #4 patients have been shown to follow a more indolent disease course than non-subset #4 *IGHV4-34* CLL¹¹⁵, while little is known about the clinical course of subset #16 patients. In contrast, subset #2 cases, which express *IGHV3-21/IGLV3-21* genes and a more or less identical 9-amino acid long HCDR3, show an equally poor survival as non-subset #2 *IGHV3-21* patients, however, subset #2 patients appear to have a shorter time to progression^{57,115,120-123}.

Several SNP-array studies have been performed in order to better understand the pathogenesis of CLL^{62,178,230}. However, limited knowledge exists regarding the spectrum of genomic aberrations in subset versus non-subset cases. Hence, we aimed to apply high-resolution 250K SNP-arrays to a total of 101 CLL cases; 17 subset #4, 8 subset #16 and 34 non-subset #4/16 as well as 29 subset #2 and 13 non-subset #2 cases, in order to investigate genomic alterations within these specific subsets.

Overall, the frequency of patients carrying CNAs was higher in subset #2 and non-subset #2 *IGHV3-21* CLL (97% and 92%, respectively) compared to subset #4 (76%), subset #16 (75%) and non-subset #4/16 (88%) samples. In subset #2 and non-subset #2 patients, we could detect the known recurrent aberrations in 90% and 54% of samples, respectively. The deletion of 11q was frequently found in both subset #2 (31%) and non-subset #2 (23%). Furthermore, concurrent 11q and 13q deletion was observed in both subset #2 and non-subset #2 although more frequently in subset #2 cases (75%, 9/12). These observations underline the adverse survival reported for

IGHV3-21 patients^{57,121,122}, in particular subset #2 patients. It is possible that these aberrations represent important events acquired during leukemogenesis in subset #2 cases, possibly in response to antigenic stimulation. In contrast, subset #4 cases displayed a lower frequency of CNAs, but also absence of poor prognostic markers. These findings may reflect the indolent disease course followed by subset #4 patients. We found subset #16 cases to carry both poor and intermediate prognostic markers.

Evaluation of genomic complexity revealed that subset #2 exhibited a higher frequency of samples (48%) with ≥ 3 CNAs in contrast to subset #4 and #16 samples (18% and 13%, respectively). The majority of patients in subset #4 carried either no aberrations or a single aberration, whereas all subset #16 patients displayed only one or two aberrations. However, no such discrete patterns could be found in the non-subset #4/16 group where all patients were observed to display CNAs ranging from 1 to ≥ 3 . Interestingly, when investigating the size distribution of CNAs, patients belonging to subset #4 did not carry aberrations > 5 Mbp, in contrast to all other subsets investigated. Accordingly, subset #4 displayed a higher prevalence of smaller aberrations in comparison to subset #2 and non-subset #2. Thus, *IGHV3-21* precursor cells appear to be more susceptible to genomic alterations than subset #4 which may be due to a more active proliferative state.

Novel aberrations on the long arm of chromosomes 2, 7 and 14 were identified in *IGHV4-34* CLL, however these were not restricted to any particular subset. On the other hand, recurrent gains on the long arm of chromosome 2 and losses at the short arm of chromosome 3 were identified in two subset #2 cases. CNN-LOH was detected on the long arm of chromosome 13 in both *IGHV3-21* and *IGHV4-34* CLL, irrespective of subset/non-subset classification, but always with concomitant homozygous del(13q). Despite not being able to discriminate between subsets based on the presence of LOH, this genomic event may nevertheless play an important role in the development of CLL although not subset specific.

In conclusion, our results from high-density screening revealed that a higher proportion of subset #2 and non-subset #2 *IGHV3-21* samples carried genomic aberrations as well as known recurrent aberrations. Considering the high frequencies of del(11q) and del(13q) in subset #2, this implies that these genetic events are important in *IGHV3-21* leukemogenesis. The particular high frequency of del(11q) in subset #2 also coincides with the shorter time to progression reported for these patients^{115,123}. On the contrary, subset #4 patients displayed a low frequency of aberrations with the absence of poor-prognostic aberrations, which in part may reflect their more indolent disease course. Hypothetically, subset #4 *IGHV4-34* CLL could be less dependent on acquiring genomic aberrations but instead more dependant on persistent (super)antigenic interaction which maintains the low-proliferative clone.

Paper II

Gene expression array characterization of stereotyped CLL subsets

The aim of this study was to investigate the global gene expression pattern in 25 *IGHV4-34* expressing patients (11 subset #4, 5 subset #16 and 9 non-subset) using sorted CLL cells and Affymetrix HG U133 Plus 2.0 arrays. We aimed to identify differences in gene expression which may provide further insight into essential biological pathways as well as the mechanisms behind CLL pathogenesis within these subsets.

When performing a first round filtering, genes with average expression intensity at background level, as well as all non-significant genes were removed from the analysis; a total of 447 significantly differentially expressed genes were identified when comparing subset #4 to subset #16. Subsequently, a second filtering step was employed where only genes having at least a 1.5 fold difference in average expression between the two groups were selected, resulting in 111 significant genes. The analysis revealed an overall lower expression of these genes for subset #4 compared to subset #16. On the other hand, evaluation of gene expression differences between subset #4 and non-subset cases, and similarly between subset #16 patients and non-subset patients, resulted in few significant differences. This may be explained by the overall observation that non-subset cases exhibited a more heterogeneous gene expression profile than subset cases.

GOTM and IPA characterization of these genes in biological processes revealed lower expression of genes involved in cell cycle control and proliferation for subset #4. It has previously been shown that CLL cells exhibit variable responses when stimulated through the BCR^{96,231}. Results from *in vitro* stimulation of their surface IG demonstrated that mutated CLL cells are less responsive than unmutated CLL^{127,128}. Subset #4 is representative of the mutated CLL group and a plausible explanation for this phenomenon may be that the clonal cells are anergized due to persistent antigenic stimulation and therefore less responsive to BCR cross-linking than other CLL subsets. Therefore, in subset #4 patients, the lower expression of genes involved in cell regulatory pathways may be attributed to the “anergic state” of this subset.

Worth mentioning is the observation that several of the differentially expressed genes that displayed a lower expression in subset #4, have previously been implicated in other cancers and cancer related pathways. Such genes include *HOXA1*, *STOML2*, *ZNF268* and *SSFA2* and over-

expression of these genes has resulted in tumorigenesis²³²⁻²³⁵. These findings may further explain the favorable prognosis evidenced in subset #4 cases.

In this study, validation of *IL15*, *HOXA1* and *ZHX1* was performed using RQ-PCR and a significantly lower expression of these genes was found in subset #4.

We also compared the expression profile for subset #4 to all other *IGHV4-34* expressing cases, however fewer differences were found. Nevertheless, subset #4 again displayed a lower expression when compared to all other cases. Several of these genes have been shown to be important in tumorigenesis including *RPS27L* and *INHBC*, particularly for their roles in p53 regulation and oncogenic transformation²³⁶⁻²³⁸.

In a recent report, an association between persistent infections by EBV and CMV and subset #4 was observed¹²⁵, here the authors alluded to the possibility that these viruses contributed to clonal expansion. Interestingly, in the present study we identified several genes involved in the facilitation of viral adhesion and viral replication to be down-regulated in subset #4 in comparison to subset #16. Speculatively, this observation may reflect an ongoing attempt by the CLL tumor cells to counteract and restrict the potential effects of viral invasion. Additionally, we have recently reported subset #4 cases to display extensive intraclonal diversification. This phenomenon was more or less unique to subset #4 cases and again strengthens the idea of ongoing persistent antigenic stimulation within this subset¹²⁶.

To summarize, in this study we were able to detect distinctly different expression profiles for subset #4 compared to subset #16 *IGHV4-34* expressing cases. We were able to identify differentially expressed genes involved in essential cell regulatory pathways including cell cycle control and proliferation; all of which displayed a lower expression in the indolent subset #4 cases compared to subset #16. Moreover, this observation of a lower expressed gene pattern in subset #4 may provide evidence for differences in the underlying biological mechanisms within these subsets. Furthermore, we also observed that non-subset cases carry a heterogeneous gene expression profile, possibly reflecting their heterogeneity and hence should be used with caution in comparative GEP. Taken together, these findings provide further evidence for biological differences in the pathogenesis of these subsets and underscore the functional significance of subset assignment based on BCR sequence features.

Paper III

Genome-wide methylation profiling in stereotyped CLL subsets

As mentioned, we recently reported the first global methylation profiling study in CLL using high-resolution methylation arrays (27,578 CpG sites) on patients belonging to the *IGHV* mutated and *IGHV* unmutated/*IGHV3-21* groups²²⁵. Significant differences in methylation patterns were observed when comparing these subgroups. For instance, several tumor suppressor genes were methylated in *IGHV* unmutated CLL, while a number of genes involved in cell proliferation and tumor progression were methylated in *IGHV* mutated CLL.

In the methylation study undertaken for this thesis, we analyzed the global gene methylation pattern in 39 CLL patients belonging to the unfavorable subsets #1 (n=15) and #2 (n=9) and the more favorable subset #4 (n=15). High-resolution 27K methylation arrays were applied with the specific aim of identifying differentially methylated genes in order to better understand the regulatory processes that might determine the pathogenesis for these stereotyped subsets.

Strict criteria were applied in order to identify differentially methylated genes between subsets (average geometric difference >0.35, p-values p<0.05 as detailed in Materials and Methods). Overall, we identified 106 genes which were significantly differentially methylated between subsets #1 and #4, while 60 genes differed between subsets #1 and #2, and 56 genes differed between subsets #2 vs. #4. Furthermore, PCA demonstrated that the CLL samples separated into three distinct clusters corresponding to subsets #1, #2 and #4. Indeed, this distinct subset clustering demonstrates that each subset displays a unique DNA methylation profile.

We also performed a comparison of the methylation profiles of subset #1 (*IGHV* unmutated) to non-stereotyped unmutated *IGHV* cases (n=8) from the previous study²²⁵. Using the same cut-off values as for the subset comparisons, we found that only 6 genes were differentially methylated. Similar results were observed when the methylation pattern of subset #4 (*IGHV* mutated) was compared to non-stereotyped mutated *IGHV* cases (n=9), again only identifying six genes displaying differences in methylation. This low level of overlapping genes may be explained by the fact that non-subset *IGHV* mutated/unmutated cases exhibit a more heterogeneous profile which may largely be due to the structural heterogeneity of their BCRs, i.e.

HCDR3s of varying lengths and no light chain restrictions. Therefore, these non-subset *IGHV* mutated/unmutated cases may recognize a wider range of antigens compared to stereotyped cases. These observations corroborate our recent GEP study, whereby non-subset *IGHV4-34* CLL patients displayed a heterogeneous gene profile whilst subset #4 CLL patients exhibited a unique profile²³⁹.

The differentially methylated genes from the three subset comparisons were subsequently grouped into distinct categories based on their biological functions such as apoptosis-related, facilitating tumorigenesis, enhancing proliferation, immune regulation, drug resistance and genes associated with prognosis. Here, comparison of subset #1 vs. subset #4 revealed several methylated pro-apoptotic genes e.g. *RIPK3*, *AATK*, *TCF3* and *IL29*, in subset #1 compared to subset #4. Moreover, the suppressor cytokine signaling 1 (*SOC31*), a known mediator of tumor suppression, was found to be hypomethylated. Interestingly, this gene has previously been reported to be silenced by aberrant methylation in AML and hepatocellular carcinoma^{240,241}. In addition, genes facilitating tumorigenesis were found to be hypomethylated in subset #1 (e.g. *WISP* and *SPN*). In contrast, when evaluating genes which differed between subset #2 and subset #4, a number of genes were found to be associated with resistance to drug therapy and/or poor prognosis (e.g. *ABCB11*, *HK2* and *ITGAM*) or previously implicated in B-cell lymphomas (e.g. *NCOR2* and *LMO2*)²⁴²⁻²⁴⁴ were methylated in subset #2.

We also found that co-stimulatory molecules CD80 and CD86 were hypermethylated in subset #1 in comparison to both subset #2 and #4. Notably, CD80 and CD86 molecules are expressed on the surface of antigen-presenting cells and interact with cytotoxic T lymphocyte antigen-4 (CTLA4). These co-stimulatory molecules play an important role in B-cell signaling and B-cell activation through the BCR, and the engagement of antigens up-regulates surface expression of these molecules. Moreover, IL-10, an autocrine growth factor required for expansion of malignant B-cells was also found to be hypermethylated in subset #1 compared to subset #2 and #4. The *IL-10* gene has previously been reported to inhibit proliferation and induce apoptosis in CLL²⁴⁵. The fact that the methylation status of *CD80*, *CD86* and *IL-10* varied at a subset level may reflect not only differences in the antigens encountered but also the environmental interactions taking place within *IGHV* mutated subset #2 and #4 compared to *IGHV* unmutated subset #1.

When performing GOTM annotation on differentially methylation genes between subset #1 vs. subset #4, we found several significantly enriched categories such as adaptive immune response, cytokine activity, T-cell differentiation and response to foreign organisms. Of particular note, was the

observation that most hypermethylated genes (75%), were found in subset #1 compared to subset #4, where they were unmethylated. In a previous study, it was reported that there is evidence for a possible link involving EBV and CMV infections in subset #4 patients. Evaluation of subset #1 and subset #2 revealed once more differential methylation of genes affecting various aspects of the immune system including the regulation of cytokine biosynthesis, T-cell proliferation and inflammatory response. In this comparison, we again noted an overrepresentation of methylated genes in subset #1. However, annotation of subset #2 and #4 revealed overrepresentation of genes involved in cytoskeletal protein binding, actin binding and transmembrane transporter activity, the pathological significance of which is currently not clear.

Validation of array data was performed on three methylated genes *ABI3*, *CD80* and *NGFR*, revealing a lower gene expression of these genes in subset #1(methylated) compared to subset #4 (unmethylated).

In summary, we demonstrate different methylation patterns when comparing stereotyped BCR subsets #1, #2 and #4. We identified differentially methylated genes that were found to be involved in important regulating pathways such as apoptosis, proliferation and the immune system. Of particular interest was the observation that genes involved in pro-apoptosis were methylated in the unfavourable subset #1, whilst genes involved in drug resistance or known to be associated with poor prognosis were unmethylated in the unfavorable subset #2. Furthermore, following functional annotation, we could observe a clear difference in the methylation of genes that are implicated in immune response when comparing subsets. These results imply that the biological differences noted between these subsets may in fact be a result of these patients encountering different antigens and illustrates an important role for epigenetic regulation in CLL pathogenesis.

Paper IV

Genome-wide methylation profiles in CLL and MCL

Previous studies focusing on methylation in MCL and CLL have largely been conducted separately, and any comparisons made have been with normal B-cells^{201,225,226}. Hence, there is limited knowledge regarding specific differences in epigenetic aberrations between these two malignancies. Therefore, this study aimed to address this issue by specifically comparing the methylation profiles in MCL and CLL. We employed high-resolution

genomic methylation microarrays (Illumina array, 27,578 CpG sites) to compare the methylation profiles in 20 MCL and 30 CLL samples. MCL patient material consisted of 10 cases with a low proliferation signature and 10 with a high proliferation signature, whereas CLL patients were represented by two prototypic subsets, *IGHV* unmutated subset #1 (poor outcome, n=15) and *IGHV* mutated subset #4 (good outcome, n=15).

We performed hierarchical clustering and PCA in order to reveal underlying methylation differences between these groups (unsupervised manner) and were able to clearly separate the DNA methylation profiles of MCL and CLL patients. Hence, this distinct separation indicates that the distribution of promoter DNA methylation is considerably different between these malignancies. In the clustering of CLL, several subgroups within CLL were noted, probably representing the heterogeneity of the disease. In contrast, MCL appears remarkably homogenous with regards to the methylation pattern as no major subgroups were observed following unsupervised hierarchical clustering.

A number of genes were found to be differentially methylated between MCL and CLL. When MCL was compared to all CLL samples (subsets #1 and #4 combined) we identified 95 genes to be differentially methylated. However, when MCL was compared to a specific subset a much larger number of genes was identified; 139 genes were differentially methylated when MCL was compared to subset #1, and 163 genes were identified when compared to CLL subset #4. Furthermore, we identified 51 genes that were present in all three comparisons, and therefore were consistently differentially methylated in MCL vs. CLL, regardless of CLL stereotypy.

We performed IPA and GOTM analysis in order to identify pathways enriched by differentially methylated loci between MCL and CLL. IPA results identified the top networks and these were shown to be involved in cell cycle control, cell death, and cell to cell signaling. Furthermore, GOTM identified homeobox and transcription factor gene promoters that were preferentially methylated in MCL compared to CLL. Among the 19 genes highly methylated in MCL, we identified six (32%) homeobox or homeodomain-containing transcription factors. This is the first study to describe extensive homeobox gene methylation in MCL.

Homeobox family genes encode DNA-binding transcription factors. Hypermethylation of homeobox genes has previously been reported in cancers of the lung, testis, and breast²⁴⁶⁻²⁴⁸. In this study, we identified six hypermethylated genes (*HLXB9*, *HOXA13*, *LHX1*, *PAX7*, *PITX3* and *POU4F1*) that belonged to the homeobox family or transcription factor genes. Three of the homeobox genes, i.e. *HLXB9*, *HOXA9* and *HOXA13*, have been identified in fusion transcripts in AML and in particular *HOXA9*

has been shown to be required for survival in MLL-rearranged acute leukemias^{249,250}.

Since several of these homeobox genes were found to be methylated, we investigated the gene expression of 4 HOX genes (i.e. *HLXB9*, *HOXA13*, *PAX7* and *LHX1*). Notably, none of these genes was found to be expressed in either MCL or CLL. A possible explanation for the silencing in CLL could be due to alternative mechanism of DNA methylation for example, histone modifications. Thus, the pathobiological significance of homeobox gene methylation in MCL must be studied further.

Interestingly, in this study, we noted that apoptosis-related genes had a high degree of methylation in CLL compared to MCL. We identified 7 pro-apoptosis-related genes to be hypermethylated in CLL (i.e. *DYRK2*, *NR4A1*, *PRKAR2B*, *CYFIP2*, *PRDM2*, *ADCYAP1* and *IL32*). Furthermore, two of these genes, *CYFIP2* and *DYRK2*, have been reported to play a role in p53 mediated apoptosis^{251,252}. Another gene, *PRDM2*, has been reported to be a tumor suppressor gene and a member of a nuclear histone/protein methyltransferase superfamily²⁵³. We also identified other hypermethylated genes in CLL, including the tumor suppressor genes *SEP9* and *PRDM2*, the cell cycle regulator *CCND1* and the histone deacetylase *HDAC11*. Furthermore, in line with our previous methylation study the tumor suppressor genes *ABI3* and *VHL* were methylated only in unmutated CLL patients (subset #1)²²⁵.

In order to validate the arrays we used RQ-PCR and found a significant difference in the mRNA levels of the *CD80* and *CAMP* gene between MCL and CLL subset patients. The *CD80* (methylated in MCL) gene was found to be lower expressed in MCL and the *CAMP* gene (methylated in CLL) was found to have higher expression in MCL compared to CLL.

In summary, this study of the methylation profiles in MCL and CLL, revealed significant differences. In MCL, the methylation profile was rather homogenous without clear formation of clusters in comparison to CLL. Furthermore, we were able to identify specific functional classes of genes that were preferentially methylated in either MCL or CLL. Importantly, homeobox genes had a higher degree of methylation in MCL compared to CLL, while apoptosis-related genes and proliferation-associated genes were methylated in CLL. Taken together, we here describe for the first time differences in global methylation profiles between these two hematological malignancies, and provide knowledge which may aid in unraveling the distinct epigenetic silencing mechanisms involved in the pathogenesis of these B-cell malignancies.

Concluding remarks

The remarkable finding of stereotyped BCRs in multiple subsets of CLL has significantly advanced our knowledge of CLL^{57,58,112,114,115}. Today, over hundred subsets has been identified, compiled from patient materials originating from diverse geographical regions¹¹⁶. However, few have investigated these subsets in terms of genetic/epigenetic alterations and gene expression profiles. In this thesis, we have employed high-resolution techniques such as GEP arrays, SNP-arrays and methylation arrays, to enable us to investigate the molecular characteristics of these CLL subsets.

In **paper I**, the aim was to investigate genomic aberrations in patients belonging to CLL subsets #2, #4 and #16 as well as their non-subset counterparts. The SNP-array screening revealed that good-prognostic subset #4 cases showed a low overall incidence of genomic aberrations compared to the poor-prognostic subset #2. Another interesting finding was that subset #4 only displayed the good prognostic marker del(13q). In contrast, the frequency of del(13q) and del(11q) was notably higher in subset #2 cases. Furthermore, these cases were found to carry a greater number of additional genomic alterations. In summary, this study illustrates the differences in genomic spectra between these CLL subsets, which are reflected by the divergent disease courses observed in subset #2 and #4.

In **paper II**, we aimed to investigate the gene expression profile in *IGHV4-34* stereotyped subsets #4, #16 and non-subset patients. In this study, we detected distinctly different expression profiles for subset #4 compared to subset #16 *IGHV4-34* expressing cases. In contrast, non-subset cases displayed a heterogeneous gene expression profile possibly reflecting their BCR heterogeneity. A number of differentially expressed genes, found to be involved in important cell regulatory pathways including cell cycle control and proliferation, were commonly down-regulated in the good-prognostic subset #4 cases, partly reflecting the indolent disease course for these patients. These findings provide evidence for differences in the underlying biological mechanisms within these subsets and furthermore underscores the functional relevance of subset assignment based on BCR sequence features.

In **paper III**, the aim was to investigate the methylation profile in stereotyped subsets #1, #2 and #4. Overall, the methylation data revealed distinct methylation profiles for all three subsets. Differentially methylated genes were found to be involved in several important cell regulatory pathways. Of particular importance was the observation of pro-apoptotic genes (methylated in subset #1), genes involved in therapy resistance (unmethylated in subset #2) and genes enhancing proliferation (methylated in subset #4). Furthermore, functional annotation of differentially methylated genes revealed an overrepresentation of important immune pathways including T-cell differentiation and response to foreign organisms. In summary, these differential methylation patterns among stereotyped subsets highlight the epigenetic disturbance involved in the pathogenesis of CLL subsets and partly explain the clinical manifestations observed.

In **paper IV**, we aimed to investigate the genome-wide methylation profiles for CLL and MCL. In this study, we identified significant differences in methylation patterns between MCL and CLL, with a more homogenous pattern evidenced for MCL in contrast to CLL cases. We found several homeobox genes to display hypermethylation in MCL compared to CLL. In addition, we observed hypermethylation of apoptosis related genes in CLL compared to MCL. In conclusion, the data presented here describes for the first time differential methylation patterns of MCL vs. CLL patients where aberrant methylation may play a critical role in disease development.

In summary, this thesis demonstrates that stereotyped CLL subsets display distinct profiles in terms of genetic aberrations, gene expression profiles and methylation patterns. These findings provide further insight into the pathogenesis of CLL at a molecular level. Furthermore, we revealed significant differences in methylation profiles between CLL and MCL which may aid in unraveling the distinct epigenetic mechanisms involved in the pathogenesis of these B-cell malignancies.

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