Analysis of Immunoglobulin Genes and Telomeres in B cell Lymphomas and Leukemias

SARAH H. WALSH
B cell lymphomas and leukemias are heterogeneous tumors with different cellular origins. Analysis of immunoglobulin (Ig) genes enables insight into the B cell progenitor, as Ig somatic hypermutation correlates with antigen-related B cell transit through the germinal center (GC). Also, restricted Ig variable heavy chain (VH) gene repertoires in B cell malignancies could imply antigen selection during tumorigenesis. The length of telomeres has been shown to differ between GC B cells and pre/post-GC B cells, possibly representing an alternative angle to investigate B cell tumor origin.

Mantle cell lymphoma (MCL), previously postulated to derive from a naïve, pre-GC B cell, was shown to have an Ig-mutated subset (18/110 MCLs, 16%), suggestive of divergent cellular origin and GC exposure. Another subset of MCL (16/110, 15%), characterized by VH3-21/VH3-19 gene usage, alludes to a role for antigen(s) in pathogenesis, also possible for hairy cell leukemia (HCL) in which the VH3-30 gene (6/32, 19%) was overused. HCL consisted mainly of Ig-mutated cases (27/32, 84%) with low level intraclonal heterogeneity, contrasting with the proposed post-GC origin, for both Ig-mutated and Ig-unmutated HCLs. For MCL and HCL, derivation from naïve or memory marginal zone B cells which may acquire mutations without GC transit are tempting speculations, but currently little is known about this alternative immunological pathway. Heavily mutated Ig genes without intraclonal heterogeneity were demonstrated in lymphoplasmacytic lymphoma/Waldenström’s macroglobulinemia (13/14, 93%), confirming that the precursor cell was transformed after GC affinity maturation. Telomere length analysis within 304 B cell tumors revealed variable lengths; shortest in the Ig-unmutated subset of chronic lymphocytic leukemia, longest in the GC-like subtype of diffuse large B cell lymphoma, and homogeneous in MCL regardless of Ig mutation status. However, telomere length is complex with regard to GC-related origin.

In summary, this thesis has provided grounds for speculation that antigens play a role in MCL and HCL pathogenesis, although the potential antigens involved are currently unknown. It has also enabled a more informed postulation about the cellular origin of B cell tumors, which will ultimately enhance understanding of the biological background of the diseases.

Keywords: B cell lymphoma/leukemia, mantle cell lymphoma, hairy cell leukemia, lymphoplasmacytic lymphoma, immunoglobulin genes, antigen selection, telomeres, cellular origin, somatic hypermutation

Sarah Walsh, Department of Genetics and Pathology, Rudbecklaboratoriet, Uppsala University, SE-75185 Uppsala, Sweden

© Sarah Walsh 2005

ISSN 1651-6206
ISBN 91-554-6217-0
urn:nbn:se:uu:diva-5748 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-5748)
To Patrick
List of papers

This thesis is based on the following papers, referred to in the thesis by their roman numerals:

*Both authors contributed equally


*Both authors contributed equally

Reprints were made with permission from the publishers.
Related papers


Abbreviations

ABC: activated B cell
AID: activation-induced cytidine deaminase
BCL: B cell lymphoma
BCR: B cell receptor
C: constant gene
CDR: complementarity determining region
CGH: comparative genomic hybridization
CLL: chronic lymphocytic leukemia
CSR: class switch recombination
D: diversity gene
DLBCL: diffuse large B cell lymphoma
dsDNA: double-stranded DNA
FDC: follicular dendritic cell
FISH: fluorescence in situ hybridization
FL: follicular lymphoma
FR: framework region
GC: germinal center
HCL: hairy cell leukemia
Ig: immunoglobulin
J: joining gene
LPL: lymphoplasmacytic lymphoma
MALT: mucosa-associated lymphoid tissue
MCL: mantle cell lymphoma
MCL-BV: mantle cell lymphoma blastoid variant
NHEJ: non-homologous end joining
NK: natural killer
RAG: recombination activating gene
Rb: retinoblastoma protein
S: switch
SHM: somatic hypermutation
ssDNA: single-stranded DNA
T\textsubscript{H}: T helper
V: variable gene
V\textsubscript{H}: variable heavy chain
V\textsubscript{L}: variable light chain
WM: Waldenström’s macroglobulinemia
Introduction

B cell lymphomas and leukemias

In Sweden, approximately 2000 individuals are diagnosed each year with malignant lymphoma or leukemia. The World Health Organisation (WHO) classification of tumors of hematopoietic and lymphoid tissues recognizes three main categories of lymphomas; B cell lymphomas (BCLs), T and NK cell lymphomas, and Hodgkin lymphoma. Mature BCLs comprise over 90% of lymphoid neoplasms and encompass a diverse range of malignancies originating from B cells, giving rise to both lymphomas and leukemias (Table 1). Tremendous clinicopathological variation exists between the different entities and the diagnosis is based on evaluation of a combination of clinical, morphological, immunophenotypic and molecular features. Classification of BCLs within the WHO scheme usually, but not always, reflects the proposed non-malignant cellular counterpart or anatomical site of origin; for example follicular lymphoma (FL) or splenic marginal zone lymphoma. The prognosis for patients varies greatly depending on the lymphoma subtype, and also sometimes on further divisions within a subtype based on immunophenotypic or molecular characteristics. This thesis focuses on BCLs, namely mantle cell lymphoma (MCL), hairy cell leukemia (HCL), lymphoplasmacytic lymphoma/Waldenström’s macroglobulinemia (LPL/WM), and to a lesser extent diffuse large B cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL) and FL. Despite the heterogeneity that exists between subtypes, a unifying characteristic is that the precursor B cell of the tumor has a unique immunoglobulin (Ig) variable heavy chain (VH) gene rearrangement which can yield valuable information about the stage of differentiation reached by the B cell prior to malignant transformation. Ig gene analysis in different entities with emphasis on the cellular origin, which is also explored from the angle of telomere length, and the issue of antigen influence in pathogenesis are the topics of this thesis.
Table 1. The most common types of B cell lymphomas in Sweden in 2000.*

<table>
<thead>
<tr>
<th>Lymphoma type</th>
<th>No.</th>
<th>% of lymphomas</th>
<th>% of BCLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td>474</td>
<td>24.7</td>
<td>30.5</td>
</tr>
<tr>
<td>Diffuse large B cell lymphoma</td>
<td>401</td>
<td>20.9</td>
<td>25.8</td>
</tr>
<tr>
<td>Follicular lymphoma</td>
<td>216</td>
<td>11.3</td>
<td>13.9</td>
</tr>
<tr>
<td>Lymphoplasmacytic lymphoma</td>
<td>92</td>
<td>4.8</td>
<td>5.9</td>
</tr>
<tr>
<td>Low grade BCL (unspecified)</td>
<td>92</td>
<td>4.8</td>
<td>5.9</td>
</tr>
<tr>
<td>High grade BCL (unspecified)</td>
<td>81</td>
<td>4.2</td>
<td>5.2</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
<td>67</td>
<td>3.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Marginal zone lymphoma</td>
<td>59</td>
<td>3.1</td>
<td>3.8</td>
</tr>
<tr>
<td>Hairy cell leukemia</td>
<td>27</td>
<td>1.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*The total number of lymphomas (n=1919) consisted of BCL (n=1553, 80.9%), Hodgkin lymphoma (n=151, 7.9%), T cell lymphoma (n=107, 5.6%) and unspecified lymphoma (n=108, 5.6%). The table shows each subtype as a percentage of total lymphomas and of B cell lymphomas. HCL is rare but the incidence is noted as this entity is included in the thesis.1

Etiology of B cell malignancies

There are different mechanisms of disrupting the normal function of genes, including genetic aberrations such as mutations, translocations and gene amplifications, and also epigenetic aberrations such as inappropriate gene silencing by promoter methylation.5,6 A particularly important tumorigenic event in BCL is the generation of chromosome translocations, and there are two main stages during a B cell’s life span in which it is highly susceptible to pre-malignant lesions; the first is during development of the immature B cell in the bone marrow when it undergoes VDJ recombination, and the second is during the response of a mature B cell to antigen which takes place in the germinal center (GC) of lymphoid tissue.7,8 Translocations in BCL usually juxtapose an oncogene from another chromosome to close proximity of the immunoglobulin (Ig) heavy (H) chain enhancer on chromosome 14, leading to overexpression of the oncogene, such as CCND1 (cyclin D1) as a result of the t(11;14)(q13;q32) in MCL,9 and the t(14;18)(q32;q31) involving upregulation of BCL2 in FL10 and up to a third of DLBCLs.11,12 A major reason why B cells are particularly prone to transformation to lymphoma or leukemia is thus as a result of differentiation stages that compromise genomic integrity, e.g. when VDJ recombination or class switch recombination occurs.7,13,14 In addition, the somatic hypermutation process in the GC reaction may aberrantly target non-Ig sequences, such as the proto-oncogenes BCL-6 and MYC.15,17 These aberrations ultimately give rise to dysregulated cellular pathways, which increase genomic instability and promote further tumorigenic events.8

Infectious agents have been linked with the development of BCLs, both viral and bacterial. Infection with hepatitis C virus increases the risk of non-
Hodgkin lymphoma, as does acquired immunodeficiency syndromes (AIDS) caused by HIV infection, although to a lesser extent since highly active retroviral therapy was introduced for AIDS patients in 1996. The immune response to \textit{H. pylori} infection is implicated in the pathogenesis of the most common type of extranodal marginal zone BCL of mucosa-associated lymphoid tissue (MALT), gastric MALT lymphoma, and antibiotic eradication of the bacterial infection often leads to tumor regression. The potential role of antigens will be discussed further in this thesis. Other than these factors, the cause of BCLs is largely unknown, and general risk factors for other types of cancer do not seem to be involved in lymphoma development, such as UV radiation exposure, obesity, or cigarette smoking. However, there may be an increased risk of FL in female smokers and certain dietary habits such as consumption of fried red meat and dairy products have been reported to increase the risk of non-Hodgkin lymphoma.

**Mantle cell lymphoma**

MCL is an aggressive lymphoma which accounts for 4% of BCLs in Sweden, and most patients succumb to the disease as there are no curative treatment options as in other BCL entities. The median age at diagnosis is 72 years and there is a gender bias with a male:female ratio of 3:1. The disease predominantly presents with widespread lymph node involvement, but involvement of spleen, bone marrow, gastrointestinal tract or peripheral blood is also common.

**Morphology, immunophenotype and postulated cell of origin**

The tumor cells are small to medium sized lymphoid cells, often with irregular nuclei, and most closely resemble GC centrocytes, hence this lymphoma was previously classified as centrocytic lymphoma. However, it was later agreed that the tumor cells were more closely related to cells of the mantle zone that surround the GC in secondary lymphoid follicles. Therefore, the lymphoma is postulated to derive from the monoclonal expansion of a mantle zone B cell. There are three main histological growth patterns; the mantle zone pattern in which the normal lymphoid structure is still intact but infiltration by tumor cells of the mantle zone has occurred, nodular - comprised of some normal architecture but also diffuse areas of growth, and finally a diffuse growth pattern whereby extensive infiltration and loss of normal structure has occurred. Immunophenotypically, MCL has the characteristics of a mature B cell with expression of surface IgM±IgD, CD19, CD20, CD22 and CD79a. In addition, MCL is CD5+, CD43+, Bel-2+, Bel-6+ (a GC marker), CD10+, and CD23+. The latter is used to distinguish MCL from CLL which has a similar immunophenotype. A predominance of surface ex-
pression of immunoglobulin (Ig) lambda (λ) light chains over kappa (κ) light chains is a common feature of MCL, which is unusual compared to normal B cells which have the opposite bias.\(^{31}\)

**Genetic aberrations**

A highly characteristic feature of MCL, necessary for diagnosis, is the overexpression of the cell-cycle regulatory protein cyclin D1. This is caused by the translocation t(11;14)(q13;q32) placing the cyclin D1 gene \textit{CCND1} within close proximity of the IgH enhancer,\(^{33,34}\) causing upregulation of cyclin D1. This proto-oncogene plays a crucial role in the cell cycle where it regulates the activity of cyclin dependant kinases 4 and 6, which phosphorylate the Rb protein and promote cell cycling. The translocation has been demonstrated to be insufficient for tumorigenesis in mouse models,\(^{35}\) and is thus thought to be one of the initiator events in MCL contributing to genetic instability. The issue of cyclin D1 negative MCL is controversial and the general consensus has been that they do not represent true MCLs.\(^{36}\) However, a recent study showed cyclin D1 negative MCLs which instead upregulated other cyclins, D2 and D3, that interact at the G1/S phase of the cell cycle.\(^{37}\)

Another well established abnormality known to occur in 20-40% of MCL is inactivation of the ataxia telangiectasia mutated (\textit{ATM}) gene, located on chromosome 11. Comparative genomic hybridization (CGH) experiments identified that approximately one third of MCL cases have a deletion of 11q22,\(^{38}\) and the region 11q22-23 was later found to contain the caretaker gene \textit{ATM}. Furthermore, it has been shown that even if \textit{ATM} is not deleted in MCL, it can be inactivated by mutations or affected by a combination of both.\(^{39}\) As ATM has a protective role in DNA repair, inactivation of this gene renders the cell particularly vulnerable to genetic instability after DNA double-strand breaks (DSBs).

CGH studies have demonstrated that MCLs have a homogeneous pattern of genetic aberrations. Characteristic regions of amplifications include 3q, 8q (which harbors the \textit{C-MYC} gene) and 15q. Recurrent losses include 13q, 1p, 6q, 9p (which harbors the INK4a/ARF locus) and 11q.\(^{38,40}\)

**Treatment and prognosis**

To date, no curative therapy exists for MCL. The median time to disease progression following treatment is 12-18 months and the median overall survival is between 3 and 4 years.\(^{3,41-44}\) Treatment with conventional chemotherapy such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) has been reported to induce higher response rates (i.e. longer time to treatment failure) when combined with anti-CD20 (“Rituximab”, R-CHOP) but there is still no improvement in overall survival.\(^{43,45}\) For younger MCL patients (diagnosed at 65 years or younger), high dose chemotherapy with autologous stem cell transplantation (ASCT) with the addition of Ri-
tuximab is a superior option which can prolong remission but again there is no evidence that it is curative. The standard treatment for older MCL patients is R-CHOP or DHAP (dexamethasone, high-dose cytarabine, cisplatin) with Rituximab. However, the ongoing search for new, more targeted therapies in MCL is crucial. Promising preliminary results have been reported with Bortezomib, a proteasome inhibitor, and with flavopiridol, which inhibits the cyclin D1 pathway.

Although the survival time of MCL patients is approximately three years, some patients can succumb to the disease within one year and others may have a more indolent course and survive for up to nine years. Corresponding with this clinical heterogeneity, variants of the disease have been described. The morphologically distinct blastoid variant (MCL-BV) is thought to correlate with a more aggressive disease, and is associated with a higher mitotic index and additional genetic abnormalities such as p53 mutations, deletions or loss of expression of p16INK4a and p21Waf1, and increased number of chromosomal imbalances and amplifications.

However, there is a lack of prognostic markers to define into which survival category a patient will fall, although a high proliferation index, as assessed by immunostaining with the proliferation marker Ki-67 is reported to have an adverse effect on survival. Recently, the global gene expression profile of MCL has been intensely studied, with different groups aiming to characterize the disease with a genetically holistic approach, and to identify subgroups with survival differences. An interesting finding to emerge from these studies is that overexpression of genes involved in promoting cell cycling is a key characteristic of MCL, and that high expression of genes coordinately involved in proliferation correlates with poor survival.

Hairy cell leukemia

HCL is a rare BCL with an indolent course and good response to treatment. The median age at diagnosis is 55 years and there is a higher incidence in males than females (5:1). Tumor cells are found in the bone marrow and spleen, but may also circulate in peripheral blood. The majority of patients present with splenomegaly and cytopenias, most commonly monocytopenia and neutropenia, giving rise to opportunistic infections. Hairy cells (HCs) have a low proliferation rate and the accumulation of tumor cells seems to be a result of unnaturally prolonged cell survival.

Morphology, immunophenotype and postulated cell of origin

The distinctive morphology of HCs enables the diagnosis, which is confirmed by the immunophenotypic profile. The cells are metabolically active, with many mitochondria and ribosomes, uncondensed chromatin, ribosome-
lamellar complexes of unknown function, and an aberrantly active process of cytoskeletal reorganization. This latter phenomenon leads to the projection of microvilli from the cell surface, giving rise to the specific morphology of tumor cells and the nomenclature.

The tumor cells display the characteristics of a mature B cell, with surface expression of CD19, CD20, CD22 and CD79a. Ig expression is most commonly IgG but expression of multiple isotypes can occur simultaneously. In contrast to MCL, HCL is negative for CD5, CD10 and CD23. However, expression of CD11c, CD25, FMC7, CD103 (B-Ly7), and positive tartrate resistant acid phosphatase (TRAP) staining are characteristic of HCL. Recently, immunostaining for annexin A1 has been reported to be specific only for HCL and will thus be useful in the future for differential diagnosis of HCL.

HCs possess the features of an activated B cell with strong expression of adhesion receptors such as CD11c (α chain of α;β; integrin, an adhesion receptor), CD25 (α chain of IL-2 receptor), and CD103 (α chain of α;β; integrin), that allow interaction with the extracellular environment. The characteristic bone marrow fibrosis in HCL is due to HCs secreting fibronectin into their surrounding environment following stimulation by extracellular factors. The HCs are also known to exert autocrine and paracrine effects by the secretion of cytokines. Thus this disease is characterized by many influences of the tumor cells on the surrounding environment, in particular the suppressive effect on normal bone marrow hematopoiesis which gives rise to pancytopenia in most patients.

The HC does not closely resemble any normal type of B cell. The WHO classification states that the cell of origin is a “peripheral B cell of unknown post-GC stage”. A recent gene expression profiling study showed that HCL has an expression profile distinct from other BCLs, and clusters with memory B cells rather than GC B cells, and another report identified HCLs as having a “spleen signature”.

Genetic aberrations
Corresponding with the indolent nature of the disease, HCL does not appear to harbor complex genetic alterations. Early cytogenetic techniques revealed aberrations (trisomy, inversions or deletions) of chromosome 5 as the most common abnormality (12 of 30 HCLs). However, CGH studies have shown that only a small proportion of HCLs exhibit genomic imbalances; 4/12 in one study and 5/20 in another. Although aberrations of chromosome 5 remain the most recurrent imbalance, it is not a defining feature of HCL since it is not consistently found. As in many cancer types, TP53 is lost in HCL, as shown by a fluorescence in situ hybridization (FISH) study of 32 HCLs. A recent CGH study of 52 HCLs also showed alterations of chromosome 5, where 19% of cases had deletions of 5q13-31, and 6% had deletions of 7q22-q35. The minimally deleted region of 7q is also shared by
splenic marginal zone lymphoma but it is unknown what gene(s) resides in this genomic region.72

**Treatment and prognosis**

The outlook for patients with HCL is good and is distinctive from other types of lymphomas and leukemias in the response to treatment. Prior to 1984, the only effective treatment for HCL was splenectomy, but the introduction of interferon alpha (IFN-α) therapy revolutionized the treatment of HCL and was considered to be the best first-line therapy.73 However, IFN-α induces a partial response in most patients but only a complete response in a small proportion, and since the 1990s, nucleoside analogues, which induce prolonged remission, have been used in HCL treatment; deoxycoformycin (“Pentostatin”) and 2-chlorodeoxyadenosine (“Cladribine”).56,74,75 In a long-term follow-up of 241 patients treated with Pentostatin, the 5- and 10-year relapse-free survival rates were 85% and 67%, respectively.56 Generally, although prolonged remissions are achieved, when relapse occurs, other nucleosides may be administered or else treatment with Rituximab.76,77

**Lymphoplasmacytic lymphoma**

The WHO classification of this lymphoma entity is LPL/Waldenström’s macroglobulinemia (WM), as the lymphoma is often associated with the clinical syndrome known as WM.3 WM is caused by an abnormally high serum monoclonal component, which is usually IgM (“M component”). Therefore the presenting symptoms for this entity may be related to the lymphoma (e.g. lymphadenopathy, organomegaly, bone marrow failure, B symptoms) or to the M component (e.g. hyperviscosity, and other complications such as anemia, neuropathy, cryoglobulinemia and amyloidosis).3,78 The diagnosis of WM can be made if bone marrow pathology shows LPL infiltration without manifestation of WM clinical symptoms.79 However, LPL cases exist with monoclonal IgG or IgA and cannot thus be classified as WM.80,81 This is an indolent disease, involving tumor infiltration of the bone marrow, lymph nodes and spleen,3 with a median age at diagnosis of 69 years.82 As the diagnostic criteria of both LPL and WM have changed so much during the past years, it is difficult to estimate median survival times, but recent studies have reported five to ten years.82-84

**Morphology, immunophenotype and postulated cell of origin**

LPL tumor cells express surface IgM and kappa light chains. The B cell markers CD19, CD20, CD22 and CD79a and also FMC7, CD25, CD27 are expressed, but CD5, CD10, CD23, CD103 and CD11c are absent.85 There is no specific distinguishing CD marker that enables the diagnosis of LPL, so essentially a process of discluding other types of lymphomas results in the
classification. The tumor tissue is composed of a mixture of plasmacytoid cells, plasma cells and small B lymphocytes. The proposed normal cellular counterpart is a B cell which has been stimulated to differentiate into a plasma cell.3

Genetic aberrations

There is a lack of information on genomic abnormalities in this lymphoma entity, with no studies performed using modern screening methods. The t(9;14)(p13;q32) involving the IgH locus and the PAX5 gene had been reported to be characteristic of LPL,86,87 but this was proved not to be the case in later reports.88,89 and there is also an absence of translocations involving the IgH locus.89 Karyotype analysis of 37 LPL/WM cases which were subdivided according to lymphoplasmacytoid, lymphoplasmacytic or polymorphous subtypes demonstrated the presence of cytogenetic abnormalities in approximately one third of cases (mostly the polymorphous subtype).90 The most common aberration found was deletion of 6q (19%),90 which was also the only recurrent imbalance (42%) confirmed in a later report.88 The long arm of chromosome 6 is frequently altered in B cell malignancies,38,91-94 but candidate tumor suppressor genes have not yet been identified.

Treatment and prognosis

Although this disease is considered incurable, remission is of prolonged duration. The treatment administered is generally chosen from one of three options; alkylating agents (chlorambucil or cyclophosphamide), nucleoside analogs (fludaribine or cladribine), or Rituximab.95 Few prospective randomized trials have been performed so the benefits of one treatment strategy over the other is not yet known. However, the use of cladribine alone or in combination with cyclophosphamide +/- the addition of Rituximab has been recently recommended as the optimum strategy for previously untreated patients, leading to overall response rates of 94%, with median overall survival of 73 months for cladribine (and median overall survival not yet reached in patients treated also with cyclophosphamide and Rituximab).96 Adverse prognostic factors reported include age ≥65, presence of cytopenias, anemia, low hemoglobin, low albumin and high β2-microglobulin.82-84 However, as recently discussed, there is currently no consensus on what factors at presentation predict the patients who will progress more rapidly.95

Diffuse large B cell lymphoma

DLBCL is a common, aggressive lymphoma involving a diffuse tumor cell infiltration of nodal or extranodal sites.3 Cases are classified as de novo (primary) or as transformed if they arise in a patient with a previous low grade malignancy such as FL. The t(14;18)(q32;q31) which upregulates the
anti-apoptotic BCL2 gene is found in up to a third of DLBCL patients, and oncogenes such as BCL6 and MYC may be aberrantly targeted by somatic hypermutation. CGH studies have demonstrated complex genetic aberrations in 67-96% of DLBCLs, including characteristic gains of chromosome X, 18q, 7 and 12, and losses of 6q. High level amplification of 18q21 correlates with high expression of BCL2, so this gene may be overexpressed by both translocations or gene amplifications. Gene expression profiling of DLBCL has recently demonstrated that this lymphoma is composed of three distinct subtypes correlating with cellular origin; GC-like, activated B cell (ABC)-like and type 3 (with unknown cellular origin). The survival differs between the groups; GC-like DLBCL patients have superior outcome with a 5 year survival rate of 60%, compared to 35% and 39% for the ABC-like and type 3 DLBCL types, respectively. Ig genes are mutated in DLBCL, with intraclonal heterogeneity in the GC-like subset but not in the ABC-like DLBCLs. The molecular classification of DLBCL was reported to be reproduced more easily using a decision tree for subgrouping based on staining with CD10, bcl-6 and MUM1/IRF4. This enabled separation of DLBCL into the two clinically relevant subgroups; GC-like and non-GC-like, with 5 year patient survival rates of 76% and 34%, respectively. The international prognostic index (IPI) is also a useful predictor of survival in this lymphoma entity. CHOP chemotherapy is the standard treatment, and while it is effective in patients younger than 60, only 40-50% of elderly patients achieve complete responses. Additionally, the somatic hypermutation status correlates with disease prognosis, with Ig-mutated CLL patients surviving approximately twice as long (10-24 years) as Ig-unmutated CLL patients (5-9 years). The Ig-mutated CLL precursor was thought to be a post-GC B cell, and the Ig-unmutated precursor a pre-GC B cell. However, it has since been revealed that all CLLs, regardless of mutation status have a homogeneous gene expression profile, and display most similarity to memory B cells rather than naive or GC B cells. Additionally, the immunophenotype of CLL cells is reminiscent of a more activated, antigen-experienced B cell, with expres-
sion of CD23, CD25, CD69 and CD79, and it has been suggested that the expression of the naïve marker CD5 is perhaps related to activation events rather than the cellular origin. The attitude towards treatment in the past has been “watchful waiting”, with chemotherapy delayed until patients reach advanced Rai or Binet stages. Although this leukemia is incurable, the use of fludarabine and Rituximab has given more promising results than treatment with alkylating agents alone.

Follicular lymphoma

This is a common low grade lymphoma in which patients present with widespread disease. Grade 1 and 2 FL patients are usually asymptomatic but have enlarged lymph nodes, whereas grade 3 is more aggressive. The median overall survival ranges from 8-12 years. FL is characterized by the t(14;18)(q32;q21) causing BCL2 upregulation, found in up to 90% of cases. The origin of FL is unambiguous - as the name implies, it derives from follicle center cells – centrocytes and centroblasts in the GC. Correspondingly, these lymphomas display heavily mutated Ig genes and are characterized by an ongoing mutation process. The changing mutational spectrum among sequential biopsies has been thought to indicate an aberrantly activated hypermutation process. However, a decrease in intraclonal variation has been observed between presentation and relapse samples from FL patients, and this could mean that clonal heterogeneity preexists and that extra mutations are not acquired over time. This is also in line with a report which showed that distinct tumor follicles are established by many tumor cells and that metastasis is caused not only by migration of one dominant clone but of many. Treatment options for FL consist of involved field radiotherapy, which may be curative for patients with early stage disease, conventional alkylator therapy for patients with advanced stage disease, and Rituximab for relapsed or refractory patients.

B cell development

The apt title of one review described lymphoid malignancies as representing the dark side of B cell differentiation, and since BCLs originate from B cells transformed at various stages of differentiation, it is necessary to discuss relevant processes of normal B cell development. As mentioned in the etiology section, there are processes in B cell maturation during which the genome is vulnerable to aberrations – to a large degree as a consequence of DNA recombination events involving double strand breaks (DSBs).
Immunoglobulin gene rearrangement – VDJ recombination

The main biological function of B cells is to be capable of recognizing antigens, and to activate the immune response in order to eliminate infections. Each B cell has a unique Ig molecule expressed on the cell surface, which in combination with a heterodimer Ig-β/Ig-α, comprises the B cell receptor (BCR). The Ig molecule is composed of four polypeptide chains, two identical heavy chains and two identical light chains (Figure 1). Both the heavy chains and the light chains have a constant (C) region and a variable (V) region; the V region of the Ig molecule is different for each B cell, and is the site involved in antigen recognition. This diversity is generated in a process of gene segment reshuffling, known as VDJ recombination, which occurs during B cell development in the bone marrow.

![Figure 1. Structure of an immunoglobulin protein.](image)

The Ig heavy chain (IGH) locus is located on chromosome 14, and has 51 functional V_H genes, approximately 27 diversity (D) genes, 6 joining (J_H) genes and 10 C_H genes. During the pro-B cell stage, V_HDJ_H recombination occurs, a reaction initiated by the protein products of recombinase activating gene 1 (RAG1) and RAG2, which bind to recombination signal sequences that flank the gene segments and introduce DNA DSBs. Intervening DNA is excised, DNA is repaired by the cellular repair machinery via the non-homologous end joining (NHEJ) pathway and the junctions of the gene segments are ligated. In the first step, a D segment recombines with a J_H segment, then a V_H segment combines with the DJ_H segment (Figure 2).
Figure 2. VDJ recombination. (Adapted from deVillartay et al.133)

Additional diversity is generated at the junctions of the VDJ segments; palindromic (“P”) nucleotides are added during the repair process, random “N-region” nucleotides may be added by terminal deoxynucleotidyl transferase (TdT) and imprecise joining at the junctions often leads to some deleted nucleotides.126,134-136 The junction encoded by N-D-N, complementarity-determining region (CDR) 3, is the most hypervariable region of the VDJ segment. The other CDR regions, CDR1 and 2 are in the VH gene segment, and the three CDRs encode the sites of the protein most involved in Ag recognition. The protein remains structurally sound due to the presence of framework regions (FRs) beside the CDRs (see figure 1). Once VHDJH recombination is completed, transcription of the entire heavy chain gene can occur. The first Cµ gene in the transcript is Cµ, and the second Cδ. Differential splicing of introns of the primary mRNA transcript results in the translation of either Cµ or Cδ.137 thus a mature B cell expresses both IgM and IgD, respectively, with identical VDJ rearrangements.

The light chains expressed by a given B cell may be encoded by either the κ or λ light chain loci, which consist of V light chain (VL), joining (JL) and constant (CL) genes. The κ light chain locus (IGK), consisting of 31-35 functional Vκ and 5 Jκ38 is located on chromosome 2, and the λ light chain locus (IGL), consisting of 29-33 functional Vλ and 4-5 Jλ39 on chromosome 22. A similar process occurs as for the IGH locus, except that there is only one recombination step; VL to JL joining. The IGK locus rearranges first to generate a functional light chain transcript. The IGL locus will recombine only if the IGK rearrangement process is unsuccessful. The percentage of κ:λ light
chain expression of peripheral blood B cells is approximately 60:40. B cell development in the bone marrow is completed after successful IgL rearrangement and the surface expression of a complete Ig molecule. The mature or naïve B cell, which is now capable of interacting with Ag, migrates to peripheral blood and subsequently recirculates between the blood, lymph and lymphoid organs.

VDJ recombination, involving DNA DSBs and rearrangement of DNA, is a dangerous process for the B cell and mistakes in the repair process can lead to chromosomal translocations. The t(11;14) in MCL is thought to occur early in B cell differentiation, before D-JH recombination, and the t(14;18) in FL is also thought to be generated during this process but at a later stage than for MCL as normal DJH segments are observed. However, these events obviously do not cripple the B cells as they continue to differentiate and exit the bone marrow, possibly already possessing one of the predisposing events to an oncogenic phenotype.

B cell response after antigen triggering

Most lymphoid tissue, such as lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT), has structures called lymphoid follicles. In the absence of Ag activation, a primary follicle comprises a network of follicular dendritic cells (FDCs) and small resting B cells, and is surrounded by a network of draining lymphatic capillaries. Ag becomes trapped within a primary follicle, and when the B cell specific for the Ag circulates through the follicle, physiological changes occur and it becomes a site for intense B cell activation (Figure 3).

![Diagram of a secondary lymphoid follicle](image)

**Figure 3.** Structure of a secondary lymphoid follicle; the outer follicular mantle surrounds the light and dark zones of the GC.
The thus called secondary follicle is composed of a ring of concentrically packed B cells (mantle zone) that surrounds the GC. The GC light zone is mainly comprised of centrocytes, and the dark zone is composed of rapidly proliferating centroblasts. In the dark zone, B cells interact with antigen presented by FDCs via the BCR and with T helper cells (T<sub>H</sub>) via the BCR and the CD40-CD40 ligand interaction. In the GC, the B cell specific for the trapped Ag undergoes clonal expansion and differentiates to form a population of Ig-secreting plasma cells and a population of memory B cells. During this process, two main events of antibody maturation occur, class switch recombination and somatic hypermutation (Figure 4).<sup>143,144</sup>

**Figure 4.** Antigen-dependant immunoglobulin diversification processes that take place in GCs; class switch recombination and somatic hypermutation. For simplicity, the class switch figure shows the five Ig classes, but in reality C<sub>GO</sub> consists of four subclasses, G<sub>1</sub>, G<sub>2</sub>, G<sub>3</sub> and G<sub>4</sub>, and C<sub>GO</sub> consists of G<sub>1</sub> and G<sub>2</sub>.

Since the discovery in 1999 that activation induced cytidine deaminase (AID) was specifically expressed in GC B cells and was crucial in both somatic hypermutation and class switch recombination,<sup>145,146</sup> rapid advances have been made in unraveling the mechanisms of these two processes, and the following sections will summarise the current hypotheses.

**Somatic hypermutation**

Affinity maturation is a process during which the binding capacity that the Ig molecule has for the Ag is altered. B cells that acquire increased Ig affinity are selected to differentiate further whereas those with reduced affinity die by apoptosis. Alteration of the germline sequence of the V(D)J segment is accomplished by the introduction of mutations which can change the amino acid sequence of the Ig protein. Repeated rounds of mutation and selection result in a clonal population of B cells, deriving from the progenitor Ag-selected B cell, that have highly optimized binding affinity for the Ag. The mutations are mainly substitutions,<sup>147</sup> accumulating at an estimated rate of approximately 1 per 1000bp per cell division.<sup>148</sup> The 5’ boundary for muta-
tions is the promoter upstream of the V(D)J segment, with mutations peak-
ing in the rearranged gene, and gradually trailing off as the distance in-
creases downstream of the V(D)J gene segment.147 The most popular model
for the mechanism of somatic hypermutation was proposed by Neuberger
and colleagues and is known as the DNA deamination hypothesis.149

AID is widely believed to deaminate cytidine residues in single stranded
DNA, resulting in a dU·dG mismatch (Figure 5). Phase 1A of somatic hy-
permutation yields transition mutations as a result of replication over the
mismatch. The next step is uracil-DNA glycosylase (UNG)-mediated exci-
sion of the dU, resulting in an abasic site. Replication at this stage – Phase
1B of somatic hypermutation – gives rise to transitions and transversion
mutations, although if the polymerase involved has a preference for purines
(dA or dG), there will be a bias towards transition mutations, which are
known to account for approx 60% of the mutations in somatic hypermuta-
tion.147,150,151 The original lesion is then resolved by apyrimidinic endonu-
clase (APE1) excision of the abasic site, followed by repair via homologous
recombination (using the undamaged sister chromatid as a template).152
Since this process is normally error-free, a highly error-prone polymerase
must be involved in the process and the polymerases thought to be involved
are pol ι (iota) and pol η (eta).153-155 Phase 2 of somatic hypermutation is
largely restricted to dA·dT mutations, and is thought to occur via
MSH2/MSH6-mediated mismatch repair of the AID-generated dU·dG mis-
mash.156,157 The mechanism for this phase of somatic hypermutation is cur-
rently unknown, but could arise by patch repair of the AID-generated dU·dG
lesion with involvement of error prone DNA polymerases, such as pol η,
having more of an impact in phase 2 rather than phase 1. Another alternative
which has been raised is that dU is occasionally misincorporated into the V₄₉
gen, leading to dA·dU pairs, which when replication occurs over the abasic
site gives rise to transition and transversion mutations.158

Regulation and specific targeting of somatic hypermutation to the Ig locus
are issues that are still unclear. Transcription of the V gene is strongly asso-
ciated with the process; when the promoter is removed the rate of mutations
is drastically reduced159 and the frequency of mutation is directly correlated
with the rate of transcription.160 The targeting of somatic hypermutation is not
dependent on the V gene,161 but is more likely linked to cis-acting factors
such as the promoter59 and the enhancer.62 Certain sequences are thought to
act as “hotspots” (RGYW or its complement, WRCY) that are specifically
targeted and mutated, during the recognition phase.163
Another pertinent question is how does AID mutate ds Ig genes when the substrate for AID is ssDNA? Experiments by Alt and colleagues have shown that AID is not sufficient by itself to drive mutation of transcribed dsRGYW motifs \textit{in vitro} and they thus set out to identify what other factors interact with AID. They recently identified replication protein A (RPA) as the complementing factor to AID,\textsuperscript{164} and proposed that AID-RPA complexes preferentially bind single-stranded transcription bubbles at hypermutation hotspots (the bubble can be as small as 4 nt). AID deaminates cytidine, and is then released leaving RPA bound which possibly is then involved in recruiting repair factors. This circumvents the problem of AID’s substrate being ssDNA, and also sheds light on the targeting mechanism. Shen and Storb recently proved that AID \textit{is} capable of targeting dsDNA but only when it is negatively supercoiled. These authors thus expand on an earlier hypothesis\textsuperscript{165} and suggest that AID associates with the transcription complex at the promoter of an expressed Ig gene and travels along with RNA polymerase II, allowing AID to gain access to altered DNA topology in the negative supercoil ahead of the replication fork.\textsuperscript{166}

\textbf{Figure 5.} Mechanism of somatic hypermutation. (Adapted from Rada \textit{et al.}\textsuperscript{151})
Class switch recombination

Class switch recombination is the change in the isotype of the Ig produced by the B cell and involves recombination between “switch” (S) regions that are located upstream from each CH region, except C\(\delta_5\), and is accompanied by the looping out and excision of intervening DNA.\(^{167}\) Replacement of the C\(\mu\) region by a C region of another Ig class (C\(\gamma\)-1-4, C\(\alpha\)-1-2 or C\(\epsilon\)) allows the expression of a different isotype, IgG1-4, IgA1-2 or IgE, respectively. IgD, as stated, is expressed via alternative splicing of the primary VDJ-recombined heavy chain RNA transcript.\(^{137}\) Different cytokines generated during the immune response to an antigen direct switching to particular CH genes, depending on the type of effector functions that are required.\(^{168,169}\)

Each of the genes for the different CH subclasses that undergo class switch are preceded by an activation/cytokine responsive promoter, an exon (I exon) and the S region.\(^{168}\) Upon cytokine (+ or – CD40 binding) stimulation, activation of transcription occurs for a certain CH gene. The process of transcription is thought to provide an opportunity for AID to deaminate dC residues leading to the generation of staggered DSBs.\(^{170}\) The S regions are composed of dG-rich highly repetitive sequence, and when transcription occurs through this sequence, it is thought that AID has access to ssDNA due to the formation of RNA-DNA hybrids (R-loops) as a consequence of the dG-rich sequence.\(^{171}\) The action of UNG and APE1, as in somatic hypermutation, generates closely spaced nicks on opposite strands.\(^{149}\) This process simultaneously occurs at a downstream S region and it is thought that the two S regions are brought together, or synapsed, and the DSB repaired with excision of intervening DNA, thus generating an S hybrid formed by the two involved S regions.\(^{170}\) The DSB is repaired via the non-homologous end joining (NHEJ) pathway\(^{172,173}\) and other proteins involved in DNA repair such as ATM,\(^{174}\) 53BP1\(^{175}\) and H2AX.\(^{176}\)

Immunoglobulin genes and B cell lymphomas

Somatic hypermutation status as a hallmark of germinal center exposure

As the presence of somatic hypermutation in V\(\text{H}\) genes is an indication that the B cell had reached or passed the GC stage of differentiation, analysis of the mutation status of V\(\text{H}\) genes in a lymphoma provides insight into the stage of differentiation that the clonal B cell had reached, prior to malignant transformation. The absence of mutations in the clonal Ig of a lymphoma or leukemia is considered to reflect that the progenitor B cell was immature or was a naïve, mature B cell that had not encountered Ag, i.e. a pre-GC B cell. Likewise, mutated Ig genes indicate a GC or post-GC stage of origin for the
malignancy. In addition, “ongoing” mutations are thought to reflect that the hypermutation machinery is still active and that Ag may be having a chronic stimulatory effect on proliferation. This is demonstrated by intraclonal heterogeneity where some of the tumor clones carry extra mutations in the V_H genes, for example in FL or GC-like DLBCL.\(^{102,120}\) However, the only way to determine if mutations are truly ongoing is to analyze multiple subclones from sequential tumor tissue from the same patient, as has been performed in FL.\(^{120,123}\) Otherwise, when looking at individual tumor biopsies that demonstrate intraclonal heterogeneity, what can be deduced is that i) they are most likely derived from the dark zone of the GC and ii) that there is a possibility that antigen stimulation is chronic or else that antigen was involved in the early stages of transformation. Mutated V_H genes are defined as deviation >2% from the published germline gene sequence. This border has been chosen in the past in order to avoid counting polymorphisms or Taq polymerase errors generated during the PCR reaction as mutations.

Figure 6 shows the postulated stage of origin of different malignancies in relation to the GC, reflecting the views at the time the work in this thesis was started. The derivation of BCLs at the GC or post-GC stage suggests that malignant transformation often occurs or possibly is initiated in GC B cells, the reasons for which have been previously discussed. Both CLL and MCL were considered to derive from naïve, pre-GC B cells, but as discussed earlier, CLL was found to consist of two subsets, 50% with unmutated V_H genes, and 50% with mutated V_H genes.\(^{107,108}\)

**Figure 6.** Origin of BCLs in relation to the germinal center. DLBCL consists of two subsets; GC-like, considered to derive from the GC and ABC-like thought to derive from a B cell that has traversed the GC. The origin of MCL indicates a pre-GC mantle zone B cell but this will be further discussed in the thesis.
Few studies had been performed in MCL, prior to the papers included in this thesis, and the results were conflicting. A report of six MCLs demonstrated no mutated cases, and a study of five MCLs reported one case with ongoing mutations but this was later queried. Five mutated cases were detected among ten MCLs, but only one of the “mutated” cases was <98% homologous to the germline gene, and five of 11 MCLs were mutated in another report. The focus for two other reports was MCL-BV; three of nine were considered mutated (96%, 97.7% and 99% homologous to the germline gene), and one of four. The disaccord between these studies was probably a reflection of the small series of MCLs analyzed and the lack of consistency in the definition of a mutated Ig gene.

For HCL and LPL/WM, these were also entities which had not been well-characterized. In HCL, one study of seven HCLs demonstrated all to harbor Ig mutations, although one case was 98.8% homologous and thus would be classified as not having undergone somatic hypermutation using the 2% cutoff. Also, since the authors subcloned cases before sequencing, they found indications of intraclonal variation in mutated cases. A report of five HCLs showed 98.6 and 98.2% homology in two cases, and <98% homology for the remaining three. This report also showed a low level of intraclonal heterogeneity. Reports of Ig analysis in small series of LPL/WM patients have shown mutated V_{H} genes and no intraclonal variation. However, the evolution of two clones in a WM patient, with the same V_{H}DJ_{H} and V_{J} junctions but different mutations has been described.

### Alternative pathways to acquire somatic hypermutation

The traditional classification of B cell malignancies with respect to their normal counterpart based on the pattern of somatic hypermutation is now not as clear cut since recent studies have revealed that it is possible for normal B cells to gain Ig mutations outside of the classical GC/T cell-dependent route, although the mechanisms by which this occurs is far from elucidated. As mentioned, B cells in extrafollicular areas of secondary lymphoid tissue are capable of responding to antigens and undergoing clonal expansion. The splenic marginal zone is such an area. The spleen has an important role in responding rapidly to blood-borne antigens, and can mount a rapid attack against polysaccharide structures on the surface of encapsulated bacteria. These type of antigens (T independent type 2 (TI-2) antigens) are capable of stimulating a B cell response in the absence of T cell help. Splenic marginal zone B cells are involved in the extrafollicular response to TI-2 antigens and the majority are memory B cells (with somatic hypermutation) with a small percentage of naïve B cells (without somatic hypermutation). Similarly, the marginal zone of lymph nodes has also been shown to display B cells with both mutated and unmutated Ig genes.
Memory B cells in the peripheral blood of a healthy adult that are IgM+IgD+CD27+ (approximately 15% of the B cell population) have been considered in the past to be conventional memory B cells that had gone through the GC reaction, as these cells harbor Ig somatic hypermutation and CD27 is a memory B cell marker. However, Weller et al showed that IgM+IgD+CD27+ B cells were the only memory (CD27+) B cells in patients with X-linked hyper IgM syndrome (HIGM1). These patients lack the capacity to form GCs due to a defect in the CD40L gene, but the IgM+IgD+CD27+ B cells in these patients still harbored somatic hypermutation in their Ig genes, indicating that it was possible for B cells to acquire somatic hypermutation without GC formation. Recently, Weller et al demonstrated that the IgM+IgD+CD27+ B cells in peripheral blood are the circulating counterpart of splenic marginal zone B cells, and that these B cells may have Ig mutations prior to differentiation in response to T-independent antigens. Interestingly, similar to possible ex-GC somatic hypermutation, the presence of switched isotypes has been demonstrated in the response to TI-2 antigens; with IgG and IgA antibodies detected in the response to capsular polysaccharide of Streptococcus pneumoniae.

These findings imply that there is a distinct B cell subset, both in the marginal zone of spleen/lymph nodes and also circulating in the peripheral blood, that has been overlooked when interpreting Ig data on B cell malignancies and may now allow a fuller understanding of their cellular origin.

Biased V_H gene usage in B cell lymphomas – evidence for involvement of antigens in pathogenesis?

Theoretically, if VDJ recombination involves random selection of V_H genes, a balanced distribution of all 51 functional V_H genes should be observed when V_H genes in B cell populations are sequenced, but in reality this is not the case. The V_H repertoire can have inherent biases in normal individuals – during the pro-B cell stage, V_H4-34, V_H4-39 and V_H4-59 gene segments are the most frequently rearranged V_H4 family genes, similarly V_H3-23 and V_H3-30 in the V_H3 family. In addition, the repertoire may change during human aging, with V_H3 members expressed more frequently in young adults and V_H4 expression observed more often in peripheral blood B cells of elderly humans. The usage of certain V_H genes has been linked with autoimmunity; V_H4-34 (previously annotated V_H4.21) with autoantibodies against red blood cell antigens, and against DNA in systemic lupus erythematosus. V_H3-21 (previously DP-77) is employed in the VDJ segment encoding antibodies against self antigens in synovial fluid in rheumatoid arthritis, autoantibodies in Sjögren’s syndrome and autoantibodies directed against platelet
glycoprotein Ia/IIa.\textsuperscript{205} $\text{V}_{\text{H}}$3-30 has been demonstrated to encode the autoantigen specificity against platelets in thrombocytopenic idiopathic purpura.\textsuperscript{206}

A restricted usage of $\text{V}_{\text{H}}$ genes has been shown in several B cell malignancies, which indirectly has indicated a role of antigen involvement in lymphoma development; B cells expressing Ig molecules encoded by certain $\text{V}_{\text{H}}$ genes may predispose to malignancy, possibly by an increased likelihood that they will be triggered to proliferate by either self or non-self antigens. Several $\text{V}_{\text{H}}$ genes, such as $\text{V}_{\text{H}}$1-69, $\text{V}_{\text{H}}$4-34 and $\text{V}_{\text{H}}$3-07, have been documented overused in CLL.\textsuperscript{207-209} The $\text{V}_{\text{H}}$1-69\textsuperscript{+} Ig rearrangements were earlier shown to display restricted characteristics such as usage of certain D and J\textsubscript{H} segments and longer than average CDR3s with specific amino acid motifs.\textsuperscript{207} Furthermore, the Ig repertoire of CLL has recently been shown to be composed of several distinct sets of characteristic $\text{V}_{\text{H}}$DJ\textsubscript{H} combinations which give rise to similar CDR3 specificities within each set, for example $\text{V}_{\text{H}}$3-21\textsuperscript{+} CLL with highly homologous CDR3s associated with the light chain gene, $\text{V}_{\gamma}$2-14.\textsuperscript{109,210,211} Since the antigen recognition site is highly conserved within these distinct characteristic Ig sets, it is conceivable that a limited number of antigenic epitopes are involved in stimulating CLL precursor cells.\textsuperscript{109,210,211} Biased usage of $\text{V}_{\text{H}}$1-69 has also been found in CLL patients with autoimmune hemolytic anemia,\textsuperscript{212} and in salivary gland MALT lymphoma.\textsuperscript{213}

There are some links between exogeneous antigens and development of B cell malignancies. Conclusive evidence is provided by the case of \textit{H. pylori} infection and gastric MALT lymphoma. In a recent study of 115 cases, 99 were \textit{H. pylori} positive, and treatment to eradicate the infection also led to complete remission of the lymphoma in 72 patients.\textsuperscript{22} Interestingly, the $\text{V}_{\text{H}}$1-69 gene is also overused in hepatitis C virus-associated immunocytoma\textsuperscript{214} (immunocytoma is now classified as LPL) and nodal marginal zone B cell lymphoma,\textsuperscript{215} and $\text{V}_{\text{H}}$1-69-encoded antibodies have been shown to be specific for the viral antigen E2.\textsuperscript{216}

In MCL, prior to this thesis, the only known overused $\text{V}_{\text{H}}$ gene segment was $\text{V}_{\text{H}}$4-34, shown by immunostaining with an anti-$\text{V}_{\text{H}}$4-34 antibody.\textsuperscript{217} Similarly, HCL and LPL/WM were poorly characterized, with Ig data published only on small cohorts of patients with no biases in the $\text{V}_{\text{H}}$ gene repertoire revealed.\textsuperscript{183,184,186,218} However, a recent report on LPL/WM described an overrepresentation of $\text{V}_{\text{H}}$3/J\textsubscript{H}4 in 11 of 15 cases, but did not indicate which $\text{V}_{\text{H}}$3 gene family members were utilized.\textsuperscript{219}
Telomeres

The ends of chromosomes are composed of long stretches of repetitive DNA (TTAGGG)_n – telomeres – which are coated with telomere binding proteins, and function together to protect chromosome ends from degradation, activation of DNA repair processes and in general, maintenance of genomic stability.220-225 The length of telomeres is approximately 5-10 kb in newly generated cells, but progressive shortening of 50-100 bp occurs with each round of cell division.226,227 When telomere length reaches a minimum threshold, cells are programmed to undergo growth arrest or apoptosis. Therefore, telomeres function as a safety mechanism to ensure that cells have a finite life span, since telomerase, the enzyme responsible for generating new telomeric DNA, is generally not expressed in somatic cells.228-235 Notable exceptions to this rule under normal circumstances are in regenerative cells, germ line cells, hematopoietic stem cells and lymphocytes.227,233,235-237 Tumor cells must overcome the regulatory barrier posed by telomere shortening in order to become immortal, and it is now well established that many types of cancer have short but stable telomeres,238 achieved by aberrant upregulation of telomerase,233,239 or in the case of telomerase negative tumors with extended telomeres, by an as yet unknown mechanism.240 However, this is a formidable research field in itself which is not the focus of this thesis and will not be considered further (reviewed by Feldser, Hackett and Greider).241

Telomeres in normal B cells

Normal lymphocytes and hematopoietic progenitor cells were found to express telomerase,236,242 which led to the hypothesis that telomerase activity facilitates rapid cell division in lymphocytes and also the capability of indefinite proliferation in order to guarantee immunologic memory. In addition, B cells are capable of upregulating telomerase expression upon mitogenic stimulation.236 Interestingly, the length of telomeres and telomerase expression differs according to the differentiation stage of the B cell.243,244-246 Thus, telomere length is shortest in naïve B cells, then as B cells enter the GC reaction and undergo clonal expansion, this enhanced proliferation is facilitated by upregulation of telomerase and lengthening of telomeres,243-245 and memory B cells have shorter telomeres than GC B cells. The difference in telomere length between naïve and memory B cells is slightly controversial, as one study described telomere restriction fragment (TRF) length to be 2 kbp longer in CD19^CD27^ cells compared to CD19^CD27^ cells,246 whereas another study reported no consistent difference in telomere length between the same subsets.247 Weng et al have reported the TRF of naïve B cells to be 7.8-8.5 kbp, of GC B cells to be 8.6-9.3 kbp, and of memory B cells to be 7.6-8.5 kbp.244 Norrback et al also char-
acterized the difference between GC B cell subsets; naïve B cells have a mean TRF of 8.51 kbp, with a significant increase to 11.42 kbp in centroblasts, and even longer telomeres, 12.45 kbp in centrocytes. This latter finding of longer telomeres in the noncycling centrocytes compared to the centroblasts probably reflects that the increase in telomerase during the centroblast stage is required for the rapid division and telomeres consequently shorten. Telomerase expression during the centrocyte stage possibly endows the cell with enhanced replicative capability to respond to future immune stimulation in the case of memory cell differentiation.

Telomeres and B cell malignancy
Corresponding with the differences in telomere length in normal B cell subpopulations, it has been recently suggested that this could also be mirrored in the malignant cellular counterparts. Analysis of telomere length in B cell malignancies (n=123) classified as GC-inexperienced, GC-derived or post-GC by Ladetto et al showed the TRF length to be highly variable. The longest telomeres were found in GC-derived entities (FL, DLBCL and Burkitt’s lymphoma) whereas shorter and intermediate length telomeres were found in pre-GC (CLL and MCL) and post-GC derived malignancies (marginal zone lymphoma and multiple myeloma), respectively. Furthermore, telomere length was shown to be a prognostic indicator in CLL in 1998 and was later shown to differ between the CLL subgroups; Ig-mutated CLL have long telomeres and Ig-unmutated cases have short telomeres. Thus, the concept that telomere length reflects histopathogenesis with respect to the GC may serve as another tool to investigate the cellular origin of B cell malignancies. However, to date only the most common entities have been analyzed, with only nine MCLs included in the Ladetto report, and HCL and LPL have never been investigated.
Aims

- To characterize the $V_{H}$ gene usage and the Ig somatic hypermutation status in MCL, in order to investigate the hypothesis that MCL derives from a naïve, pre-GC B cell, and also to determine if antigen selection has a role in the pathogenesis of the lymphoma.

- To perform Ig analysis in HCL and LPL/WM, both of which are B cell malignancies which have previously not been extensively investigated, in order to gain insights into the cellular origin and $V_{H}$ gene repertoire.

- To analyze the cellular origin of different B cell lymphoma and leukemia entities by comparing differences in telomere length which is known to differ in normal B cell populations in relation to the GC.
Methods

Patient material
All patients included in the studies were diagnosed according to the criteria in the WHO classification for each disease. Detailed information regarding each cohort can be found in papers I-V. A total of 110 MCL patients have been analyzed in papers I and II, and a selection of 73 cases were included in paper IV. The t(11;14) or cyclin D1 protein overexpression has been confirmed in all MCLs, and clinical information collected for 109 patients. The median age at diagnosis was 68 years and the median overall survival was 3 years. HCL samples from 32 patients were analyzed in paper III, and 19 of the 32 HCLs were further investigated in paper V. The median age at diagnosis for the 19 HCL patients was 56 years and the median overall survival was 19 years. Paper IV consists of the analysis of 14 LPL/WM cases, of which seven were included in paper V, with the addition of two new cases. The median age at diagnosis of the 14 LPL/WM patients was 72 years, and the median overall survival was 5 years, all cases were CD19+/CD20+/CD22+/CD5-/CD10-/CD23-/κ+. In paper V, the cohort of 101 de novo DLBCLs had a median age at diagnosis of 66 years, with a median overall survival of 3 years, and the 76 CLLs all had Binet stage A disease, and a median overall survival of 9 years. Twenty-six FLs were also included in paper V, in addition to the MCL, HCL and LPL cases. Clinical data and survival information were obtained from medical records and local Swedish cancer registries.

Ig gene analysis
DNA was prepared from tumor material using standard protocols or with the QIAamp DNA Mini Kit. VH and VL gene family-specific PCR amplification was performed as outlined in paper I and II. In summary, clonal products from the VH or VL gene PCR were either sequenced directly using the BigDye Terminator Cycle Sequencing Reaction Kit or PCR products were subcloned using the Zero Blunt TOPO PCR Cloning kit prior to sequencing in order to investigate intraclonal heterogeneity, germline gene sequences in T cell DNA or else performed if direct sequencing was problematic. All sequences were analysed using an automated DNA sequencer (ABI 377 or ABI 3700). The obtained sequences were aligned to Ig sequence databases; GenBank (National Center for Biotechnology Information, USA), V-BASE (MRC Center for Protein Engineering, Cambridge, United Kingdom) and
International ImMunoGeneTics (IMGT). V\textsubscript{H} gene sequences carrying mutations resulting in <98% homology to the germline gene were defined as mutated. The presence of mutations in hotspot regions was evaluated by analysing the number of mutations that occurred in the germline sequence motif RGYW/WRCY. In general, intraclonal heterogeneity was defined as the presence of an extra replacement mutation shared by at least 2 of 10 subclones analyzed, but for paper III, mutations were classified as shared (by all clones), partially shared (by at least two clones) or unique.


telomere length analysis

The telomere length of tumor DNA was measured by quantitative PCR according to Cawthon with minor modifications. Relative telomere length was measured by determining the factor by which the sample analyzed differed from a reference sample in its ratio of telomere (T) repeat copy number to a single (S) copy gene number (β2-globin). The cycle threshold (Ct) is lower if telomeres are longer since there are an increased number of binding sites for primers to extend from, resulting in higher T/S ratios. Ct values for telomere amplification and β2-globin amplification were calculated for each sample. The standard curve for each PCR was generated using reference DNA (CCRF-CEM cell line). This reference DNA was also used to normalize T/S values so that samples from different runs could be compared. Both reference and sample DNA were analyzed in triplicates. The Ct values generated in both runs were used to calculate T/S values for each sample:

\[
T/S = 2^{-\Delta C_t} \text{ (where } \Delta C_t = C_{t_{control}} - C_{t_{telomere}})\]


Statistical analyses

Statistical tests were conducted to investigate if there was skewing of distribution of various parameters in different groups. Tests used included the Fisher’s exact test with one-tailed p-value, the McNemar Chi-square test, the ANOVA test and the Mann Whitney U test. Patient survival was calculated from the date of diagnosis until the last follow up or death. Kaplan-Meier survival analysis and log-rank test were performed to study the prognostic impact of various parameters on patient survival in different entities and subgroups. Multivariate analysis was used to study the prognostic strength of univariately significant parameters. In all tests, P values less than 0.05 were considered to indicate significant differences. Statistica 6.0 software (StatSoft Inc, Tulsa, OK, USA) was used for all calculations.
Results and Discussion

**Papers I and II**

Paper I comprises the analysis of IgH gene rearrangements in 51 MCLs, representing the largest such study in MCL at that time, and demonstrated the presence of mutated Ig genes in a sizeable proportion (n=10, 20%) of MCL cases. On the basis of this data, we proposed that MCL consists of two subsets; one with unmutated V\textsubscript{H} genes originating from a pre-GC B-cell, and the other with mutated V\textsubscript{H} genes deriving from a B-cell exposed to the GC environment. There was no increased frequency of blastoid variant MCL (n=2) compared to classic MCL (n=8) in the 10 mutated cases, of note since a study of nine blastoid variant cases reported three to be characterized by Ig mutations.\textsuperscript{181} We found no preliminary evidence for intraclonal variation but the number of subclones sequenced was small (between 3 and 6). Furthermore, there was no difference in survival between Ig-mutated vs Ig-unmutated MCL, as in CLL.\textsuperscript{107,108} However, we wished to extend the analysis and characterize MCL in greater depth based on the new findings in paper I. The study was expanded to 110 patients in paper II and hence these results will be discussed in greater detail.

Somatic hypermutations were present in the Ig genes of 18 MCLs (16%), which contrasts with the proposed pre-GC derivation of MCL. However, the mean mutation percent of 3% (range 2.2-6.7%) is quite low in comparison with the mutation load in GC-derived entities (mean 9% in FL).\textsuperscript{121} Therefore sequencing of the corresponding unrearranged gene in germline DNA from 5 patients with Ig-mutated MCL displaying 2-3% mutations was performed and this confirmed that the mutations were only present in the V\textsubscript{H} gene of the lymphoma DNA (Figure 7). This was an important finding which demonstrated that although the mutation load was low, it still constituted hypermutated Ig genes. This was further substantiated by analyzing the number of mutations that were in the known somatic hypermutation hotspots, which revealed that 50% of the mutations fell in these regions (total number of mutations from all mutated cases, 121, of which 60 were in RGYW/WRCY motifs). However, there was no survival difference between patients on the basis of presence or absence of Ig mutations (18 vs 39 months, respectively, $P=0.28$). The issue of intraclonal variation was investigated in five Ig-mutated cases, but no evidence of heterogeneity was found, which led us to suggest that MCL with mutated Ig genes, although they possibly have been
exposed to the GC, they do not appear to be GC-derived. However, as the suggestion that an alternative pathway exists in which hypermutation can be acquired in the absence of GC formation has been raised, it cannot thus be ruled out that mutated MCL precursors arise from this subset of B cells that acquire somatic hypermutations via a different, as yet uncharacterized pathway.

![MCL no. 51 clonal Ig rearrangement](image)

**Figure 7.** Comparison of lymphoma DNA with clonal Ig rearrangement and germ-line DNA (from T cells) from the same patient. The six mutations in the rearranged gene (97.3% homology to the V H3-23 germline gene) were absent in unrearranged germ-line DNA, demonstrating that the mutations in MCL were due to somatic hypermutation. This was true for the other four cases analyzed in the same manner.

In paper I, there was an overusage of V H4-34 (n=11, 22%), V H3-21 (n=8, 16%) and V H5-51 (n=6, 12%). Analysis of the larger material showed that the most prevalent V H genes in the MCL repertoire were V H3-21 (n=21, 18%), V H4-34 (n=19, 16%), V H3-23 (n=9, 8%), V H5-51 (n=8, 7%) and V H1-8 (n=7, 6%). The patients with V H3-21+ MCL had superior overall survival compared to the remaining patients (53 vs 34 months, \( P = 0.03 \)), whereas no difference in survival was correlated with usage of the other frequently utilized V H genes. Interestingly, all but one of the V H3-21+ patients expressed \( \lambda \) light chains and we therefore sequenced the V \( \lambda \) gene rearrangements and found that most cases utilized the V \( \lambda 3-19 \) gene (16 of 18 amplifiable V L genes). It must be stressed that to find the same combination of V H and V \( \lambda \) gene segments in 15% of MCL is highly significant considering the random combinatorial events during VDJ rearrangement of the IGH locus and VJ rearrangement (in IGK initially and then IGL only if the \( \kappa \) light chain rearrangement is unsuccessful). The V H3-21 genes were all unmutated, therefore B cells bearing V H3-21-encoded Ig may, hypothetically, be aberrantly stimulated to proliferate by antigen via a T cell-independent pathway. The combined usage of V H3-21/V L3-19 suggests a possible role for antigen(s) in the pathogenesis of these tumors and indicates that V H3-21+ patients constitute a new MCL subgroup.

Other reports have since confirmed our findings; Ig-mutated MCL has been reported to constitute 28% of MCLs by Camacho et al., and 29% by Kienle et al. The latter study also showed that for both Ig-unmutated and
Ig-mutated MCL, there was no evidence of isotype class switch or BCL-6 expression, which supports a non-GC-derivation for the mutated cases. Antigen involvement in MCL is also proposed by Kienle and coworkers, since VH3-21+ cases, predominantly in the Ig-unmutated subset, have highly characteristic rearrangements with long CDR3s and use of the D3-3 and Jh6 gene segments. 255

In conclusion, papers I and II revealed more molecular heterogeneity in MCL and enabled the proposal of new MCL subsets. The Ig-mutated MCL subset may have an alternative origin than a naïve, pre-GC B cell. The subset characterized by overrepresentation of the VH3-21/V\(\gamma\)3-19 combination fuels speculation that MCL-genesis may be partially antigen-driven.

Paper III

Ig analysis of 32 HCL cases was performed in this study, and somatic hypermutation was detected in the clonal Ig genes of 27 cases (84%), with a mean percentage mutation of 5%. Subcloning of eight Ig-mutated cases revealed intraclonal variation in all cases. The number of extra mutations that were shared by at least 2 subclones for each case was zero for three cases, and 2-3 for the remaining five cases, but there was a large number of unique extra mutations among clones. Thus, intraclonal variation exists in this leukemia, but at a lower level as compared to GC-derived follicular lymphoma.121 These findings indicate that Ig-mutated HCLs have a closer association with the GC than previously thought, and do not have a true post-GC B cell derivation. This interpretation is supported by the finding of multiple clonally related isotypes on both the mRNA and protein level,61,184 which has prompted the suggestion that HCLs are derived from a post-somatic hypermutation, pre-isotype class switch B cell. However, expression profiling has shown that HCLs are most similar to memory B cells, and also are characterized by a spleen expression profile signature.67,68 Of the five cases which were Ig-unmutated, four were 100% and one was 98.4% homologous to the germline gene. This smaller subset of patients with unmutated Ig genes has been identified also in a cohort of 13 patients, in which five cases were >98% homologous to the germline gene.256 A post-GC B cell may thus not be the origin for Ig-unmutated HCL, unless the precursor B cell underwent the GC reaction without acquiring somatic hypermutation. A more plausible scenario is that these cases may derive from a naïve splenic marginal zone B cell. Indeed, for both subsets of HCL the cellular origin may be marginal zone B cells, with the unmutated and mutated cases deriving from naïve and memory marginal zone B cells, respectively.

A biased usage of the VH3-30 gene was detected in this material; utilized in the V\(\gamma\)DJ3 rearrangement in 6 of 32 cases (19%). This high proportion of VH3-30, all of which displayed somatic hypermutations, may imply antigenic
selection and malignant transformation of B cells with V_{H}3-30-encoded Ig molecules. The lack of characteristic CDR3 structure or specific associations with D and J_{H} segments may entail that other parts of the Ig molecule, such as CDR1, CDR2 or the framework regions are more important for interaction with antigen. Thus, a superantigen or autoantigen could be triggering proliferation of B cells with V_{H}3-30-encoded Ig molecules in the splenic marginal zone. The association of V_{H}3-30 with an autoimmune disorder (idiopathic cytopenic purpura) reinforces this suggestion. In a recent report, V_{H}3-30 was not found overused in a series of 20 HCLs (2/20), in which the most frequently used V_{H} genes were V_{H}3-23, V_{H}3-07 and V_{H}3-33, and larger cohorts will be needed to confirm this bias. However, we hypothesize that an antigen with specificity for V_{H}3-30-encoded antibodies could be involved in HCL.

In conclusion, a post-GC B cell of origin is now questionable for HCL, both for the Ig-mutated cases with intraclonal heterogeneity, which fits closer with a GC origin, and also for the Ig-unmutated cases. The high percentage of HCL with clonal V_{H}3-30-encoded Ig rearrangements could implicate antigenic involvement in disease pathogenesis.

**Paper IV**
Fourteen LPL/WMs were investigated in paper IV. Although a relatively small cohort, it is a difficult lymphoma to collect large materials of due to the classification difficulties associated with exclusion criteria. Most investigated patients had a serum IgM component of variable magnitude, while one patient had an IgG component and another both IgG and IgM. Ig gene analysis demonstrated the presence of a high number of mutations in 13 cases (mean mutation 8%), whereas a germline configuration was found for the remaining case. The question of intraclonal variation in LPL/WM was resolved in this study by sequencing many subclones (up to 27 clones), which showed that LPL/WM tumor clones are homogeneous. This confirms the postulated cell of origin as being a B cell stimulated to differentiate to a plasma cell. A pre-isotype class switched B cell has been suggested to be the LPL precursor, a hypothesis supported by the majority of cases producing/secreting IgM, and also by the lack of detection of switch transcripts. However, considering the fact that some cases do secrete other isotypes, and that the extent of IgM secretion can vary so widely between different patients, we hypothesize that individual LPL cases are derived from B cells at various developmental stages between a late GC B cell and a plasma cell. Speculatively, the rare Ig-unmutated cases may derive from a plasma cell of the primary immune response.

No biases were found in the V_{H}, D or J_{H} gene repertoires in this entity, with no V_{H} gene used more than twice, and similarly a random use of D and J_{H} genes. Thus, we could not confirm the recent findings by Kriangkum et al.
who analyzed 15 LPL/WM cases and reported a strong biased use of \(V_{\text{H}3}/J_{\text{H}4}\) gene family members. However, they have not stated if the individual \(V_{\text{H}}\) or \(J_{\text{H}}\) genes used were the same and thus it is hard to draw many conclusions from their finding.\(^{219}\) Although biases may not be apparent in smaller cohorts of patients, this lymphoma entity does not appear to be characterized by a restricted \(V_{\text{H}}\) gene repertoire.

**Paper V**

Telomere length was reported to correlate with histopathogenesis of B cell malignancies in a recent study of 123 samples, in which short, long and intermediate telomere lengths were observed in pre-GC, GC and post-GC-derived tumors, respectively.\(^{248}\) In this report, DNA from 304 B cell tumors was analyzed for telomere length by quantitative PCR, which measured the ratio between telomere (T) copies and single (S) copy gene for each sample. Low T/S values indicate short telomeres while high T/S values indicate longer telomeres. The different entities analyzed were DLBCL (n=101), CLL (n=76), MCL (n=73), FL (n=26), HCL (n=19) and LPL/WM (n=9). The median T/S value for all 304 cases was 0.47 (range 0.03-9.49), but differences existed between the overall entities (Table 2); median T/S values from shortest to longest were as follows; CLL (0.35), MCL (0.46), LPL (0.49), FL (0.51), DLBCL (0.54) and HCL (0.59). However, more pronounced differences were found when lymphoma entities were subdivided. Ig mutation status was used to separate CLL, MCL and HCL into subgroups. CLL, as previously described,\(^{248,250,251,258}\) had significantly different telomere length in the Ig-mutated subset (0.62) compared to the Ig-unmutated subset (0.20) \((P<0.001)\), but MCLs had homogeneous telomere length regardless of mutation status. Thus, if Ig-mutated MCL has a different cellular origin compared to Ig-unmutated MCL, this was not demonstrable on the basis of telomere length. Although in HCL the number of unmutated cases (n=4) compared to mutated cases (n=15) was low, we found no significant difference in telomere length, again suggestive of a homogeneous disease. DLBCL was separated into GC-like (n=52) and non-GC-like (n=49) cases based on staining patterns with CD10, bcl-6 and MUM1/IRF-4 as described,\(^{103,259}\) and a striking difference was found with GC-DLBCL displaying longer telomeres (0.73) compared to non-GC-DLBCL (0.44) \((P=0.0005)\), further emphasizing the heterogeneity between these subsets. However, although longest telomeres were found in GC-DLBCL, the fact that GC-derived FL had shorter telomeres (0.51) (also shorter than HCL, 0.59), prevents simple delineation of malignancies with respect to GC origin on the basis of telomere length. Survival analysis of DLBCL, CLL and MCL showed a significant association with T/S values and prognosis only in CLL; higher T/S value than the median for CLL (0.35) correlated with better sur-
vival than lower T/S value, \(P=0.03\), but was not as strong a prognostic factor as mutation status, \(P=0.0007\).

These results support the separation of DLBCL with regard to GC histopathogenesis, as recently proposed. However, although telomere length is variable and can reveal differences in subsets of B cell malignancies, such as in DLBCL and CLL, and also show the homogeneity between putative subsets, such as in MCL and HCL, it does not clearly correlate with cellular origin with respect to the GC. Confounding factors may be that the cell of origin for some entities is not fully clarified and that different proliferation rates (+/- telomerase activity) among BCLs heightens the complexity.

Table 2. Summary of Ig mutation status, intraclonal heterogeneity, mean percent somatic hypermutation (SHM) and range, \(V_\text{H}\) gene bias and telomere length in different subtypes of BCL.

<table>
<thead>
<tr>
<th>BCL</th>
<th>% mutated</th>
<th>Intraclonal variation</th>
<th>Mean % SHM</th>
<th>(V_\text{H}) gene bias</th>
<th>Tel length (T/S) median</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCL</td>
<td>16</td>
<td>NO</td>
<td>3 (2.7)</td>
<td>(V_{\text{H}}3-21, V_{\text{H}}4-34)</td>
<td>0.46</td>
</tr>
<tr>
<td>HCL</td>
<td>84</td>
<td>YES</td>
<td>5 (2.9)</td>
<td>(V_{\text{H}}3-30)</td>
<td>0.59</td>
</tr>
<tr>
<td>LPL/WM</td>
<td>93</td>
<td>NO</td>
<td>8 (4-13)</td>
<td>NO</td>
<td>0.49</td>
</tr>
<tr>
<td>CLL</td>
<td>~50</td>
<td>NO</td>
<td>6 (2.13)</td>
<td>(V_{\text{H}}1-69, V_{\text{H}}4-34, V_{\text{H}}3-07, V_{\text{H}}3-21)</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ig-unmut CLL: 0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ig-mut CLL: 0.62</td>
</tr>
<tr>
<td>DLBCL</td>
<td>~98</td>
<td>YES†</td>
<td>11 (2-27)</td>
<td>NO</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GC-DLBCL: 0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>non-GC-DLBCL: 0.44</td>
</tr>
<tr>
<td>FL</td>
<td>100</td>
<td>YES</td>
<td>9 (4-13)</td>
<td>NO</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Data based on papers I-IV, and other published literature. †: in GC-like DLBCL but not ABC-like DLBCL. \(^{102,107,119-122,207-211,260}\)
Concluding remarks

The work in this thesis has shown more molecular heterogeneity in MCL and HCL than previously thought, describing both Ig-unmutated and Ig-mutated subsets, raising the question of cellular origin in these entities, and also showed biased $V_{H}$ gene repertoires for both diseases, which is suggestive of a link between the structure of the Ig molecule and pathogenesis. In addition, it has also confirmed that mutated Ig genes with lack of intraclonal heterogeneity characterize the Ig repertoire in LPL/WM, and demonstrated that there is no $V_{H}$ bias. The issue of telomere length in different entities has highlighted the heterogeneity of BCLs and reinforced the differences between Ig-mutated and unmutated subsets of CLL and between GC-like and non-GC-like DLBCL, and also shown the homogeneity of MCL regardless of Ig mutation status. However, telomere length is not a completely clear-cut parameter to consider cellular origin with respect to GC-derivation.

Since the studies that comprise this thesis have been carried out, emerging data (mentioned in the introduction) has shown that there could be possible alternative processes in the immune system which allow B cells to acquire Ig gene mutations, without transit through the GC. The processes are far from elucidated but nevertheless allow speculation if B cell malignancies with unclear cellular origin derive from less characterized or overlooked B cell compartments. With this in mind, and taking into account data from gene expression profiles, some new speculations can be discussed which have not been raised in the published work.

Expression profiling of MCL has suggested derivation from a more activated, proliferative cell than a resting mantle zone B cell, and this can also be debated due to the identification of MCLs with mutated Ig genes. It is still unclear from where mutated MCLs arise, but the fact that no biological differences have been uncovered between the two subsets of MCL also raises the argument that all MCLs could have a similar origin, and could it then instead be that unmutated or mutated MCLs derive from naive or memory marginal zone B cells, respectively? This may be an option, especially considering that some marginal zone B cells have somatic mutations and others do not.

The origin of HCL does not seem to be a conventional post-GC B cell, both for the small subset of patients with unmutated Ig genes and also for the mutated cases which display intraclonal heterogeneity. Gene expression profiling of HCL has shown similarities to both memory B cells and
splenic B cells. Thus, Ig analysis and expression studies, taken together with the recent finding that lymphomas (eg. splenic marginal zone lymphomas) can display intraclonal variation without a GC derivation, a naive (for Ig-unmutated HCL) or memory (for Ig-mutated HCL) B cell located in the splenic marginal zone seem to be likely candidates for the cellular origins of this leukemia. Figure 8 illustrates the cellular origin of B cell malignancies with a more open-minded approach, taking into account the work in this thesis and other recent studies.
A

Classic follicular T-cell dependent antigen response

B

Extrafollicular response involving T-independent antigens
Figure 8. Reconsideration of the cellular origin of B cell lymphomas and leukemias.

**A)** The classic GC reaction and the proposed malignant counterparts. MCLs have been thought to originate from naïve B cells residing in the mantle zone surrounding GCs, and while this could well be for cases with unmutated Ig genes, it is uncertain for the subset with mutated Ig genes. CLL had also been thought to originate from naïve B cells but the presence of Ig mutations in 50% of cases, and the homogeneous expression profile of all CLL cases suggests that the origin could be a memory B cell, either a conventional post-GC B cell or from IgM⁺IgD⁺CD27⁺ memory B cells (8, part b). FL and the GC-like subset of DLBCL are GC-derived, with mutated Ig genes and an ongoing hypermutation process. LPL/WM could hypothetically undergo malignant transformation at any stage along the pathway from a late GC B cell to a plasma cell. Non-GC-like DLBCLs (ABC like and type 3) have somatic hypermutation of the Ig genes and originate from a post-GC B cell but of unknown differentiation.

**B)** The extrafollicular response to antigen. The marginal zone B cells of the spleen play an important role in this response, and the peripheral blood counterpart of these cells (IgM⁺IgD⁺CD27⁺) are thought to be memory B cells which have not traversed the GC reaction but instead harbor Ig mutations prior to antigen contact. The splenic white pulp is composed of the periarteriolar lymphoid sheath, the marginal zone and lymphoid follicles (the figure depicts two primary follicles and two secondary follicles). Splenic marginal zone lymphoma, of which ~50% harbor Ig mutations, have been suggested to derive from naïve or memory marginal zone B cells depending on mutation status. These are also tempting candidates for the precursor to the HCL subsets. For MCL, speculatively these could also derive from naïve and memory marginal zone B cells or their circulating counterparts. In addition, the overrepresentation of unmutated VH gene segments, such as VH3-21 and VH4-34 in MCL, may imply that these cases originate from a B cell undergoing a T-cell independent response in which no somatic hypermutation takes place.
Popular summary

Cancer is a disease which, after heart disease, is the second leading cause of death among the elderly in developed countries. There are many different types of cancer and this thesis is about cancer that starts off, or originates, from cells of the immune system; B CELLS, causing lymphoma or leukemia. The general difference is that lymphoma usually first manifests itself in lymphoid tissue (i.e. lymph nodes), whereas the tumor cells usually build up in the bone marrow and bloodstream in the case of leukemia.

B cells have long and interesting life spans, during which they pass through many stages of development. Thus, at any given time an individual has many different types of B cell populations in their body. B cells have an important function – they are responsible for identifying and fighting foreign substances in our body, collectively termed ANTIGENS. Every single B cell which comes out of the bone marrow into the bloodstream is unique because on the outside, covering the surface of the cell, are many identical copies of a unique protein known as the antibody or IMMUNOGLOBULIN molecule which recognizes the antigen. B cells thus search out if antigens are present, and when a B cell whose immunoglobulin matches and structurally fits with an antigen (like a lock and key), then a series of events unfolds to destroy the antigen. An important part of this antigen-B cell interaction is the formation of a GERMINAL CENTER, a place in a lymph node where B cells activated by antigen develop further. In the germinal center, CLONAL EXPANSION of the stimulated B cell takes place, which means that the B cell makes many copies of itself. Therefore, some of the key stages in a B cell’s life are 1) birth in the bone marrow 2) exit from bone marrow into blood, and movement back and forth between blood and lymph nodes 3) development in the germinal center after they meet antigen and 4) final development when the B cell makes many copies of itself to produce plasma cells which secrete free antibody molecules into the blood or else memory B cells which live for a long time and can activate a faster response to the same antigen if it comes back again. Then we can say roughly that B cells can be separated into 1) B cells which have never met antigen i.e. B cells which are pre-germinal center, 2) highly activated germinal center B cells and 3) plasma cells or memory B cells i.e. post-germinal center which have been through the germinal center reaction.

What does this have to do with lymphoma and leukemia? Different types of lymphoma/leukemia behave very differently; some are aggressive and
incurable (mantle cell lymphoma) whereas some are associated with very long survival times for patients (hairy cell leukemia). In this thesis, the type of B cell from which different types of lymphoma and leukemia start from has been studied, the “cellular origin”. This was done by looking for the presence of SOMATIC HYPERMUTATION in the immunoglobulin gene in tumor B cells because this happens to B cells when they are in the germinal center. Therefore, the presence of somatic hypermutations is like a stamp on a passport that shows that a B cell passed through the germinal center. Thus, different lymphomas and leukemias are classified as originating from a pre-germinal center, germinal center or post-germinal center B cell.

Another question asked is if antigens play a part in triggering the beginning of cancer in certain B cells. An obvious thought is why would this be happening if the normal function of B cells is to react to antigens? However, it has to be remembered that in cancer, cells do not function normally. In the case of normal B cells, they are stimulated to grow, or clonally expand, by antigens which bind to their immunoglobulin molecule. In the case of lymphomas, they arise because of abnormal clonal expansion of one starting B cell. Thus a lymphoma tumor in one patient will be made up of many identical copies of the same B cell. Then, if you look at tumor tissue from many different lymphoma patients, you would expect to see many different types of immunoglobulin structures (which we have looked for by sequencing the immunoglobulin gene that makes up the immunoglobulin protein on tumor tissue for each patient). It follows on then, that if you repeatedly see that one general immunoglobulin structure (or immunoglobulin gene used) is found in tumor tissues from many different patients, then this is indirect proof that a particular antigen might select normal B cells with that general shape immunoglobulin to grow. This could then possibly increase the likelihood that cancer events might take place.

In this thesis, we found that mantle cell lymphoma does not only originate from a pre-germinal center B cell as had been thought, because we could find somatic hypermutation of the immunoglobulin gene in tumor tissue from some patients. Therefore, we concluded that not all mantle cell lymphomas have the same cellular origin – that some patients have mantle cell lymphoma which started from a B cell that had never been through the germinal center, whereas some patients have tumors which started from a B cell which had somatic hypermutations (and thus originated from a different type of B cell). We could also reason that the immunoglobulin structure in tumors of some patients had maybe been a factor involved in development of the disease. This is more complicated to grasp but could mean that the same type of antigen in different patients had stimulated that person’s B cells which had the matching immunoglobulin fit for the antigen, to grow and somehow begin the cancer process. Maybe the antigen kept on persistently stimulating the B cell and eventually the normal function of the B cell was lost. In hairy cell leukemia, which is a tumor thought to start off in a B cell which has
passed the germinal center (post-germinal center B cell) and then should obviously have somatic hypermutations, we found that most cases did indeed have somatic hypermutations but that the pattern of mutations was more like it could possibly still be getting new hypermutations. Therefore we think that instead of a post-germinal center B cell, some cases of hairy cell leukemia start off in a cell which is more like a germinal center B cell. Also, a few hairy cell leukemia cases were found which did not have somatic hypermutations which contradicts that the starter cell had passed through the germinal center. In a few cases, like in mantle cell lymphoma, we also think that maybe an antigen played a part in stimulating the starter cell of hairy cell leukemia. We also analyzed a lymphoma called lymphoplasmacytic lymphoma which is thought to start off in a B cell which is on the way to becoming a plasma B cell (one that secretes immunoglobulin into the bloodstream), and we confirmed this. There was no sign that antigens could be involved in the cancer process.

We then tried out a new angle to look at the type of B cell that different lymphomas originated from. This was done by looking at a different factor which can change in different types of normal B cells – the length of TELOMERES. Telomeres are the name for the type of DNA that protects chromosomes in our cells from damage, they are like an overcoat for our DNA. But the function of telomeres was not the focus, we were interested in telomeres because they are known to be short in pre-germinal center B cells, very long in germinal center B cells and intermediate length in post-germinal center B cells. So could analyzing the telomere length in lymphoma tissue be another way to judge the type of B cell that the lymphomas started from? We looked at the most common types of lymphomas, which are all classified as originating from different types of B cells. We found that the telomere length was different in the different types of lymphoma and leukemia. For mantle cell lymphomas, which we earlier suggested is made up of two subtypes with different cellular origins, we found that telomere length was much the same in DNA from all cases (medium length). Therefore telomere length does not show if it is true that different mantle cell lymphomas originate in different types of B cells. For hairy cell leukemia, the relatively long telomeres in all cases also showed the overall similarity in the disease. However, known differences in disease subtypes of chronic lymphocytic leukemia and diffuse large B cell lymphoma were indeed confirmed by analysis of telomere length in tumor DNA. Overall, telomere length is interesting in B cell lymphomas and leukemias but is a little too complicated to draw parallels between a type of lymphoma and its corresponding normal B cell.

In summary, this thesis has investigated the cellular origin of B cell lymphomas and leukemias, and shown new subtypes within mantle cell lymphoma and hairy cell leukemia. There is a possibility that these tumors start off from B cells which do not go through the well-known B cell life span, but instead come from B cells that deviate from the normal B cell pathways.
This thesis has also suggested that one reason why cancer could perhaps begin, or further develop, in some of these lymphomas is because of possible abnormal responses to antigen. What is the relevance of these studies? Does this change anything for the average lymphoma patient who walks into the oncology clinic? Not directly, but it is so important to understand the biology of the diseases, including what type of B cells they originate from. Then we can try to understand what happened in the B cell to start the cancer process. Only in this way can new, specialized treatments be developed to combat these diseases.

Sarah Walsh, 31st March 2005
sarahhelen.walsh@gmail.com

GLOSSARY:

B CELLS: also known as B lymphocytes, these cells are involved in the fight against antigens.

ANTIGENS: any structure that the human body recognizes as foreign. Can be parts of viruses, bacteria, fungi, and also can be something in your own body that the immune system mistakenly thinks is foreign (which is what causes autoimmune diseases).

CLONAL EXPANSION: cells “grow” by cell division. This is when one cell splits itself into two new cells, effectively cloning itself. When a B cell is stimulated, it starts to clonally expand; therefore it is converted into two new cells, each of which do the same (so then there are 4 cells), each of which do the same (so then there are 8 cells) and so on. In cancer, a similar process occurs, so a cell which has lost normal function begins to clonally expand, eventually leading to a lot of cells which results in a tumor.

IMMUNOGLOBULIN: the molecule on B cells, also called an antibody, that can bind to antigens. It is made up from one variable gene, of which there are 51 in our DNA that a B cell can choose to use, and other genes. Since there are so many different genes that a B cell can choose to use to make up its own unique immunoglobulin, the structure of the immunoglobulin is always different (page 21 in this thesis).

GERMINAL CENTER: a structure in a lymph node where B cells fight antigens (page 23).

SOMATIC HYPERMUTATION: when a B cell is involved in fighting antigens in the germinal center, the immunoglobulin gene is targeted by mutations which change the DNA and therefore the structure of the immunoglobulin protein is also changed (for better or worse fit with the antigen) (page 24).

TELOMERES: in our cells, chromosomes are basically tightly packaged DNA so that the DNA manages to fit in the cell. The ends of chromosomes are capped with protective DNA called telomeres. Telomeric DNA is gradually eroded the more times a cell divides, and only certain cell types are capable of adding on more telomeric DNA. Germinal center B cells are such a cell type and thus their telomeres are longer compared to other types of B cells.
Acknowledgements

This work was carried out at the Department of Genetics and Pathology and Department of Oncology, Radiology and Clinical Immunology, Rudbeck Laboratory, Uppsala University. Financial support was provided by the Swedish Cancer Society and Lion’s Cancer Research Foundation, Uppsala.

I would like to sincerely thank my supervisor, Richard Rosenquist Brandell, for many aspects over the past few years; for the introduction to the research field, for being a truly dedicated supervisor, for the right balance of guidance when it was needed but then the freedom to work more independently. It has been a great experience working together, both scientifically and on a personal level.

To my co-supervisors, Christer Sundström and Anna Laurell, I would like to extend my gratitude. Anna, the insights into the clinic were very much appreciated! Christer, for sharing his expert knowledge of lymphoma pathology. I would also like to thank Gunilla Enblad as a member of the Oncology group who is also instrumental in facilitating the research at our corridor.

The members of the lymphoma research group and people at the oncology corridor at Rudbeck Lab. Mia T and Ulf T for the productive work on projects together, and the good times and laughs we had inside and outside of the lab, it has also been fun traveling together (Mia – as we enjoyed total luxury in the South of Italy, but also supported each other in less than idyllic conditions in San Diego! 😊, Ulf – I don’t think I will ever forget the quest for the dancing chicken in New York! Or the tie-breaker for the quiz you made at your summer house party…). Marie F, thanks for all the coffee and chats together, and the nice time we had at the course in Switzerland. Other members, Ingrid T, Daniel M, Mattias B, Majlis B, Martin S, Fredrik Q, Ingrid G, Jacob B. Fiona and Åsa, for chats, friendship and evenings out.

People that I have collaborated with, especially Pawel Grabowski and Göran Roos at Umeå University and Emma Flordal Thelander, Svetlana Lagercrantz, Birgitta Sander and Erik Björck at the Karolinska Institute.

To the patients who consented to research analysis of tumor material, the studies would not have been possible otherwise. Hopefully this thesis con-
tributes to scientific knowledge that may lead to better treatment options in the future.

Other staff at Rudbeck Lab; the nice ladies in administration—especially Eva Bäck and Gunilla Åberg, the IT staff, Viktor and Per-Ivan. The staff at the Hematopathology Dept. in C5, especially Simin Tahmasebpoor for always being helpful when the time came to locate samples and freeze our fingers in the biobanks!

I wish to thank my lecturers at the School of Biological Science, Dublin Institute of Technology; Brid Ann Ryan, Louis Armstrong, Ray Ryan, Derek Neylan, Fergus Ryan, Jacinta Kelly and Helen Lambkin for providing a high standard of education in biochemistry and molecular biology.

Thanks to the lovely people I have met in Sweden, both in Rudbeck Lab and outside. David T (for summer BBQs with Mia and the kids), Petter (two-meter Peter!) for the welcome to Sweden, Marcin and Kaska, Alvaro and Patricia, Linda J, Daniel Ö, the Stockholm lads Jim and Shane, Ingrid N, Ola S, Cecilia, Arek, Magda, Caisa, Caroline, Kiran, Uwe, Ricardo, Jan D, Lotta T, Malin E, Fredrik C, Marina, Arzu, Marian N and Peter R.

To Margaret, it does not feel like nearly five years since we first got chatting (for hours and hours!) but you are a great friend and we have experienced a lot together. Looking forward to spending time together back in Ireland for a change!

To everyone back home, thank you for your support, my relations in Skerries – especially Nana, Fiona and Pat, Tricia, Terry and the rest of the Byrne and Walsh families. All of Patrick’s family, the Buckleys – Ann, Ger, Aidan, Martin and Carly.

A special mention to acknowledge my wonderful grandparents who have since passed away but who were always so proud and supportive of me, Helen and Des Walsh, and Andrew Byrne.

My family; Mum and Dad, and my brothers and sisters; Peter, Katie, Rachel, Samuel and Anna-Sue. I feel so blessed to have such a loving family. Mum and Dad thank you for so much support during the years and always encouraging me to do the best I can. You are all just so good to me. (kiss kiss hug I love you all!)

To Patrick, the most special person in the world, thank you – you are a star and I couldn’t have done this without you. I love you with all my heart and soul. xxx
References


116. Byrd JC, Rai K, Peterson BL, et al. Addition of rituximab to fludarabine may prolong progression-free survival and overall survival in patients with previ-


166. Shen HM, Storb U. Activation-induced cytidine deaminase (AID) can target both DNA strands when the DNA is supercoiled. Proc Natl Acad Sci U S A. 2004;101:12997-13002.


260. Ek S, Hogerhorst CM, Dictor M, Ehinger M, Borrebaeck CA. Mantle cell lymphomas express a distinct genetic signature affecting lymphocyte trafficking


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 29

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine").