DOI:10.14753/SE.2012.1744

SOMATIC HYPERMUTATION OF IgV_H GENES AND ABERRANT SOMATIC HYPERMUTATION IN FOLLICULAR LYMPHOMA WITHOUT *BCL2* GENE REARRANGEMENT AND EXPRESSION

Ph.D. Doctoral Dissertation

Éva Gagyi, M.D.

School of Ph.D. Studies of Semmelweis University

Doctoral School of Pathological Sciences

Tutor: András Matolcsy, M.D., Ph.D., D.Sc.

Official academic reviewers: Hajnalka Andrikovics, M.D., Ph.D.
András Kiss, M.D., Ph.D.

President of the Ph.D. examination board: Janina Kulka, M.D., Ph.D.
Members of the Ph.D. examination board: Mónika Csóka, M.D., Ph.D.
Gábor Mikala, M.D., Ph.D.

Budapest

2012

DOI:10.14753/SE.2012.1744

TABLE OF CONTENT

DOI:10.14753/SE.2012.1744

DOI:10.14753/SE.2012.1744

I. ABBREVIATIONS

A -	Adenine
AID -	activation-induced (cytidine) deaminase
ALL -	acute lymphoblastic leukemia
APE -	apurinic-apyrimidinic endonuclease
ASHM -	aberrant somatic hypermutation
BCL2 -	B-cell CLL/lymphoma 2 oncoprotein
BCL6 -	B-cell CLL/lymphoma 6 oncoprotein
BER -	base excision repair
BL -	Burkitt lymphoma
BLAST -	Basic Local Alignment Search Tool
BM -	bone marrow
bp -	base pair
C -	Cytosine
CD -	cluster of differentiation
CDR 1, 2, 3 -	complementarity determining region 1, 2, 3
CLL -	chronic lymphocytic leukemia
C_T -	cycle threshold
CSR -	class switch recombination
D -	"Diversity" immunoglobulin genes
DAB -	3-3' - diaminobenzidine
DEPC -	diethyl-pyrocarbonate
DLBCL-	diffuse large B-cell lymphoma
DNA -	deoxyribonucleic acid
dNTP -	deoxy-nucleotide triphosphate
ddNTP -	dideoxy-nucleotide triphosphate
E.coli -	Escherichia coli bacteria
EDTA -	ethylene-diamine tetraacetic acid

DOI:10.14753/SE.2012.1744

EPP - error-prone polymerase

FISH - fluorescence *in situ* hybridization

FDC - follicular dendritic cell

FL - follicular lymphoma

FR - framework region

FRET - Fluorescence Resonance Energy Transfer

G - Guanine

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GC - germinal centre

GTP - guanosine-5'-triphosphate

HL - Hodgkin lymphoma

HRP - horseradish peroxidase

Ig - immunoglobulin

IgH - immunoglobulin heavy chain gene

IgV_H - "Variability" region of immunoglobulin heavy chain gene

IMGT - International imMunoGeneTics information System

J - „Joining" immunoglobulin genes

LN - lymph node

LSI - locus specific indicator

MALT - mucosa-associated lymphoid tissue

$MgCl_2$ - magnesium chloride

MM - multiple myeloma

MMR - mismatch repair

MRD - minimal residual disease

mRNA - messenger RNA

MSI - microsatellite instability

NCBI - National Center for Biotechnology Information

NHL - non-Hodgkin lymphoma

NK cell - natural killer cell

PB - peripheral blood

DOI:10.14753/SE.2012.1744

PBS -	phosphate buffer saline
PCR -	polymerase chain reaction
Q -	quencher
Q-RT-PCR -	quantitative real-time polymerase chain reaction
R -	reporter
R -	mutation causing amino acid replacement
RNA -	ribonucleic acid
rpm -	revolutions per minute
RT -	reverse transcription
S -	silent mutation, no amino acid replacement
SDS -	sodium-dodecyl-sulphate
SHM -	somatic hypermutation
T -	Thymine
Taq -	Thermus aquaticus
TCR -	T-cell receptor
T_H -	T-helper cell
TAE -	Tris base, acetic acid and EDTA
TBS -	Tris buffered saline
TE -	Tris-EDTA
UNG -	uracil-DNA glycosylase
V -	“Variability” immunoglobulin genes
WHO -	World Health Organization
X-Gal -	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

DOI:10.14753/SE.2012.1744

II. LITERATURE REVIEW

1. Introduction

Lymphoid tumours, B-cell and T/NK-cell neoplasms, are clonal tumours of mature and immature B-cells, T-cells or natural killer (NK) cells at various stages of differentiation. Mature B-cell neoplasms comprise over 90% of lymphoid neoplasms worldwide. They represent, with increasing incidence, approximately 4% of new cancers each year. They are particularly common in developed countries, particularly the United States, Australia, New Zealand and Western Europe [1].

1. 1. Cellular origin of B-cell lymphomas and B-cell lymphoma classification

The major principle of the lymphoma classification is the recognition of distinct disease entities according to a combination of morphology, immunophenotype, genetic, molecular and clinical features. The disease entities are stratified according to their cell lineage and, additionally, their derivation from precursor or mature lymphoid cells [2].

B-cell neoplasms tend to mimic stages of normal B-cell differentiation, and the resemblance to normal cell stages is a major basis for their classification and nomenclature [1]. Figure 1 shows a diagrammatic representation of B-cell differentiation and relationship to major B-cell neoplasms. B-cell development is initiated in the primary lymphoid organs with further differentiation in secondary lymphoid tissues. During these stages of development, several DNA modifications occur that are essential for a normal immune response. However, these modifications might also predispose to genetic abnormalities leading to lymphoma evolution [3].

Normal B-cell differentiation begins with progenitor B-cells, which undergo immunoglobulin V (variable) D (diversity) and J (joining) gene rearrangement. B lymphoblastic leukemia/lymphoma (B-ALL) is a neoplasm of precursor cells committed to the B-cell lineage, with germline *Ig* genes [4].

Antigen-induced B-cell activation, particularly after repeated antigen challenge, generates secondary lymphoid follicles with germinal centres (GC). During the GC reaction at least two distinct DNA modifications – somatic hypermutation (SHM) and class switch recombination (CSR) – occur. They are responsible for generating high-affinity antibodies of different subclasses that are capable of mediating specific immune responses [5]. The most common tumours of GC B-cells are follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), Burkitt lymphoma (BL) and Hodgkin lymphoma (HL) [6-9].

After the GC reaction, B-cells develop into memory B-cells and plasma cells. The most common representatives of tumours of this stage are multiple myeloma (MM) and chronic lymphocytic leukemia (CLL) [10, 11].

Although, B-cell neoplasms in many respects appear to recapitulate stages of normal B-cell differentiation, some common forms, like hairy cell leukemia, do not clearly correspond to a normal B-cell differentiation stage. Thus, the normal counterpart of the neoplastic cell cannot be the sole basis for the classification [1].

Traditionally, classical Hodgkin lymphomas have been considered separately from so-called “non-Hodgkin lymphomas” (NHL). However, with the recognition that HL is of B-cell lineage, greater overlap has been appreciated between HL and many forms of other B-cell malignancies [1, 7, 12].

Over 90% of lymphoid neoplasms are mature B-cell tumours. The most common types of mature B-cell lymphoma are FL and DLBCL, which together make up more than 60% of all lymphomas exclusive of Hodgkin lymphoma and multiple myeloma [1].

DOI:10.14753/SE.2012.1744

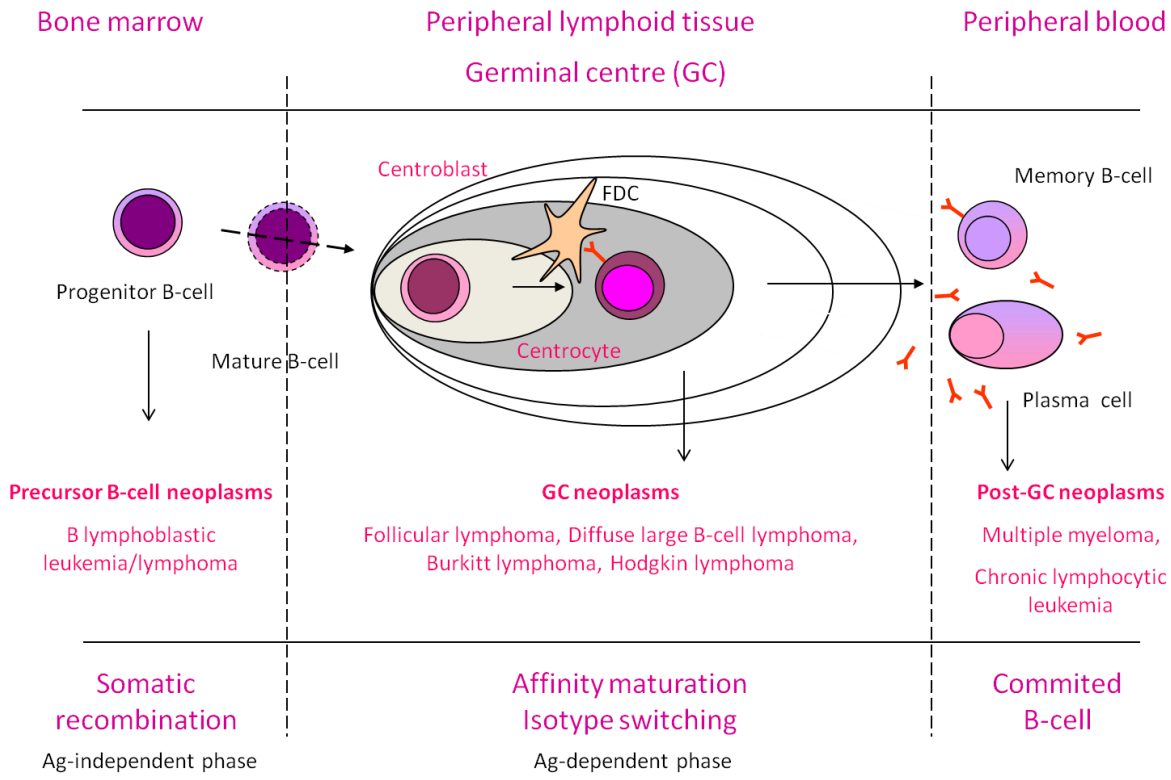

Figure 1: Diagrammatic representation of B-cell differentiation and relationship to major B-cell neoplasms. Lymphomas arise at different stages of B-cell differentiation. The first step, undertaken by bone marrow-derived B-cell precursors, is the recombination of *Ig* genes to generate a functional B-cell antigen receptor. This initial step occurs prior to antigen encounter. B lymphoblastic leukemia/lymphoma is a neoplasm of precursor cells. Antigen-induced B-cell activation initiates GC reaction in secondary lymphoid tissues and DNA modifications, somatic hypermutation (SHM) and class switch recombination (CSR) in B-cells. Interactions with the antigen presented by follicular dendritic cells results in the affinity maturation/selection of clonal B-cells.The most common neoplasms of GC B-cells are follicular lymphoma, diffuse large B-cell lymphoma, Burkitt lymphoma and Hodgkin lymphoma. After the GC reaction, B-cells develop into memory B-cells or plasma cells. Common post-GC neoplasms are multiple myeloma and chronic lymphocytic leukemia. *(FDC: follicular dendritic cell)*

DOI:10.14753/SE.2012.1744

2. Follicular lymphoma

2. 1. Epidemiological and clinical aspects of follicular lymphoma

Approximately 95% of all malignant non-Hodgkin lymphomas are – at least in the Western hemisphere – of B-cell origin. Among more than 30 different entities and subtypes of B-cell NHL, according to the World Health Organization (WHO) classification of lymphoid tumours, FL constitutes the second most common B-cell malignancy [13]. The highest incidence can be observed in the USA and Western Europe. In Eastern and Central Europe, Asia and in developing countries the incidence is much lower [14]. It affects predominantly adults, with a median age of 59 years and a male: female ratio of 1:1.7 [15]. FL rarely occurs in individuals under the age of 20 years, and paediatric patients are predominantly males [16-19].

Most patients have widespread disease at diagnosis, including peripheral and central (abdominal and thoracic) painless lymphadenopathy and splenomegaly. The bone marrow (BM) is involved in 40-70%. Implication of non-hematopoietic extranodal sites, such as the skin, gastrointestinal tract, central nervous system, ocular adnexa, breast or testis, is relatively uncommon. Despite widespread disease, patients are usually otherwise asymptomatic. Only one third of patients are in Stages I or II (Ann Arbour Staging), the majority of them present with the advanced stages III or IV at the time of diagnosis [20, 21].

In general terms, FL is considered an indolent lymphoma with a clinical evolution that is characterized by slow progression over many years. However, the clinical course of FL patients can be surprisingly variable and, accordingly, treatment options range from a ‘watch and wait’ approach to aggressive therapy [13].

Transformation from FL into an aggressive lymphoma, usually DLBCL, takes place in 25-30% of cases, which leads to clinical progression and decreased overall survival [13, 22, 23].

2. 2. Morphological features of follicular lymphoma

Most cases of FL have a predominantly follicular pattern with closely-packed follicles that efface the nodal architecture. Neoplastic follicles are often poorly defined and usually lack clear-cut mantle zones. In contrast to reactive germinal centres where centroblasts and centrocytes occupy different zones (polarization), in FL the two types of cells are randomly distributed, and often display a monomorphic appearance due to the lack of characteristic 'starry sky' pattern. Similarly, tingible body macrophages, characteristic of reactive GCs, are usually absent in FL [13, 20].

Diffuse areas – parts of the tissue completely lacking follicles defined by CD21+/CD23+ follicular dendritic cells (FDC) – may be present, often with sclerosis. Distinction between an extensive interfollicular component and a diffuse component may sometimes be arbitrary. Diffuse areas containing predominantly centrocytes are thought to be clinically insignificant, whereas diffuse areas comprised entirely or predominantly of centroblasts is equivalent to DLBCL [20].

The pattern of FL is given after the relative proportions of follicular and diffuse areas (Figure 2). The tumour is considered follicular with >75% follicular areas, follicular and diffuse with 25-75% follicular areas, and focally follicular/predominantly diffuse if has <25% follicular areas.

2. 3. Grading of follicular lymphoma

Based on the proportion of centroblasts within the malignant follicles, FL are divided into grade 1 (predominantly centrocytes), grade 2 (centrocytes and centroblasts), grade 3A (predominantly centroblasts, but centrocytes are still present) and grade 3B (solid sheets of centroblasts) categories (Figure 3) [20]. In grade 1 and 2 FL, the number of centroblasts does not exceed 150 per 10 high power fields. Grade 3A FL present with more than 150 centroblasts per 10 high power fields [24]. A number of studies suggest that this histological grading predicts clinical outcome. Cases with elevated large cell

fractions (centroblasts) behave more aggressively and show a higher likelihood of progression into DLBCL than those with fewer large cells [25-28].

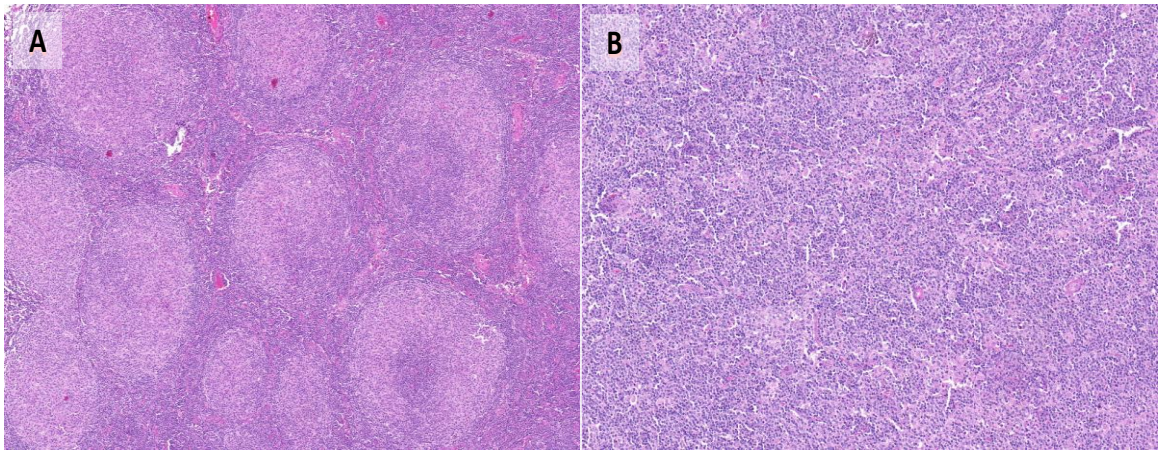

Figure 2: Follicular lymphoma. A: FL with follicular pattern. The neoplastic follicles are closely packed, focally show an almost back-to-back pattern, and lack mantle zones. Centrocytes and centroblasts are randomly distributed and the follicles display a monomorphic appearance due to the lack of characteristic 'starry sky' pattern; **B:** FL with diffuse pattern, completely lacking follicles.

2. 4. Immunophenotype of follicular lymphoma

The neoplastic cells resemble normal follicular centre B-cells, expressing surface immunoglobulin – mainly IgM but rarely also IgD, IgG and IgA – , CD19, CD20, CD22, CD79a. They are BCL2+, BCL6+, CD10+, CD5- and CD43-. Some cases, especially grade 3B, may lack CD10, but retain BCL6 expression. CD10 expression is often stronger in the follicles, than in the interfollicular neoplastic cells, and BCL6 is also downregulated in the interfollicular areas. Meshworks of FDC are present in follicular areas and may variably express CD21 and CD23 [20]. BCL2 protein is expressed by a variable proportion of the tumour cells in 85-90% of cases of grade 1 and 2 FL, but only 50% of grade 3 FL using standard antibodies. In some cases undetectability of BCL2 protein is due to mutations in the *BCL2* genes that eliminate the epitopes recognized by the most commonly used antibody, and can be detected using antibodies to other BCL2

epitopes. The proliferation index in FL generally correlates with the histologic grade, lower grades having a lower Ki67 expression (Figure 4, Figure 5 and Figure 6) [20].

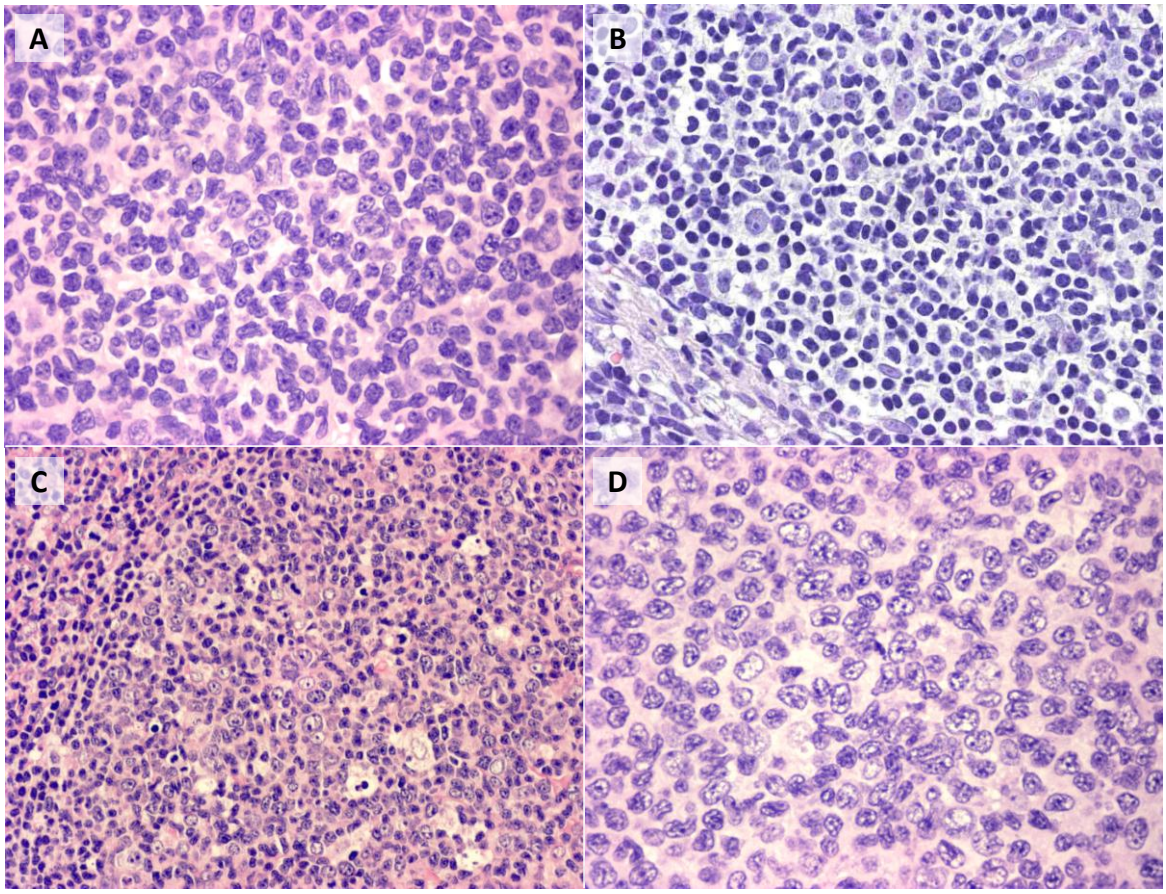

Figure 3: Follicular lymphoma grading. A and B: In FL grade 1 (A) and grade 2 (B) there is a monotonous population of small cells with irregular nuclei (centrocytes), and with only rare large cells (centroblasts) with enlarged nuclei, prominent nucleoli and abundant basophilic cytoplasm. **C:** In FL grade 3A there are more than 15 centroblasts per high power field, but centrocytes are still present. **D:** In FL grade 3B the majority of cells are centroblasts. Hematoxylin & Eosin (H&E) staining.

DOI:10.14753/SE.2012.1744

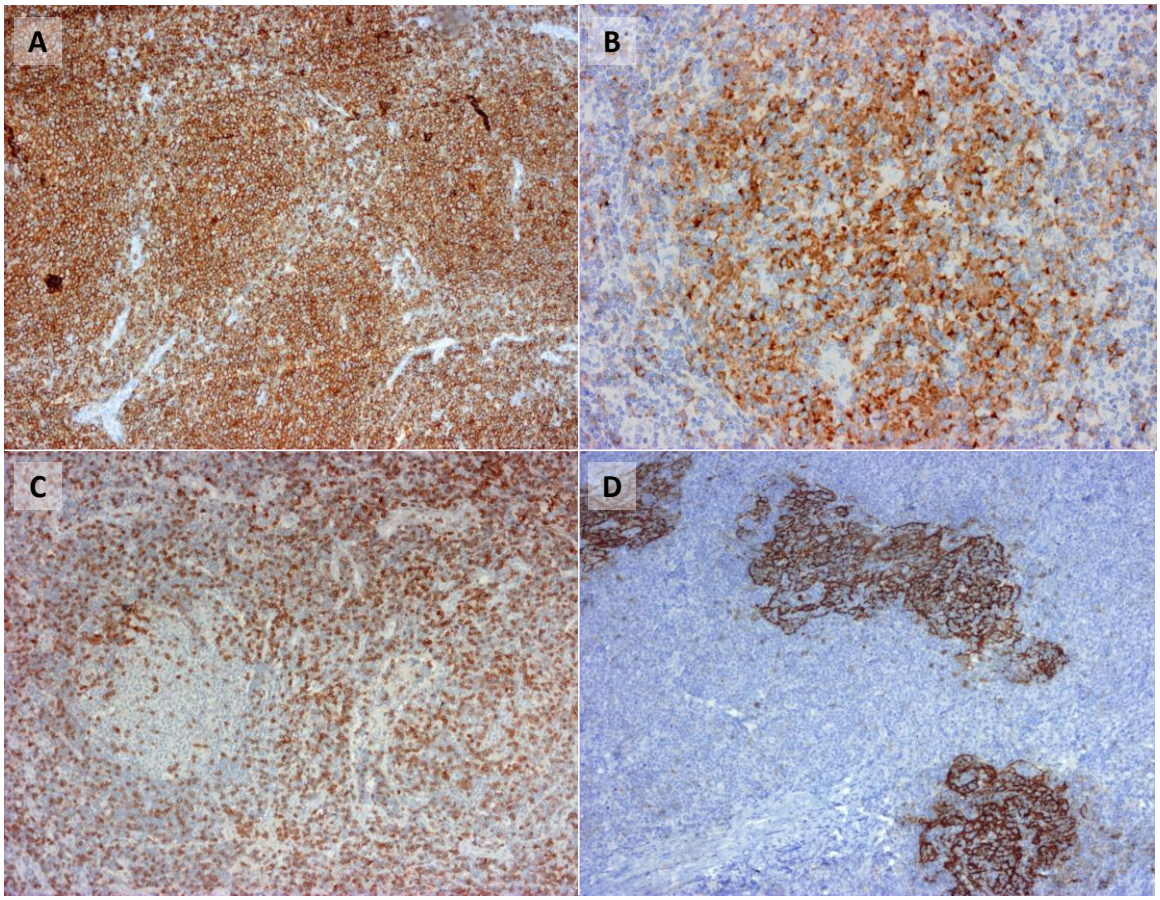

Figure 4: Immunophenotype of follicular lymphoma. Transformed follicles are enriched in CD20+ (**A**) and CD10+ (**B**) tumour cells of B-cell origin, and are surrounded by CD3 positive T-cells (**C**). CD21 reaction highlights FDC meshwork and follicular structure.

2. 5. Genotype of follicular lymphoma

In the bone marrow B-cell precursors assemble the immunoglobulin heavy (H) and light (L) chain genes by somatic recombination to generate functional B-cell antigen receptor. The recombination activity targets the variable (V), diversity (D) and joining (J) regions of *IgH* (Figure 7) [29]. Upon antigen encounter, during the immune response, B lymphocytes undergo somatic mutations. In the GC many non-random, single-base changes are introduced by SHM into the *IgV* regions – mainly in the form of single base substitutions, with insertions and deletions being less common – that encode the antigen-

DOI:10.14753/SE.2012.1744

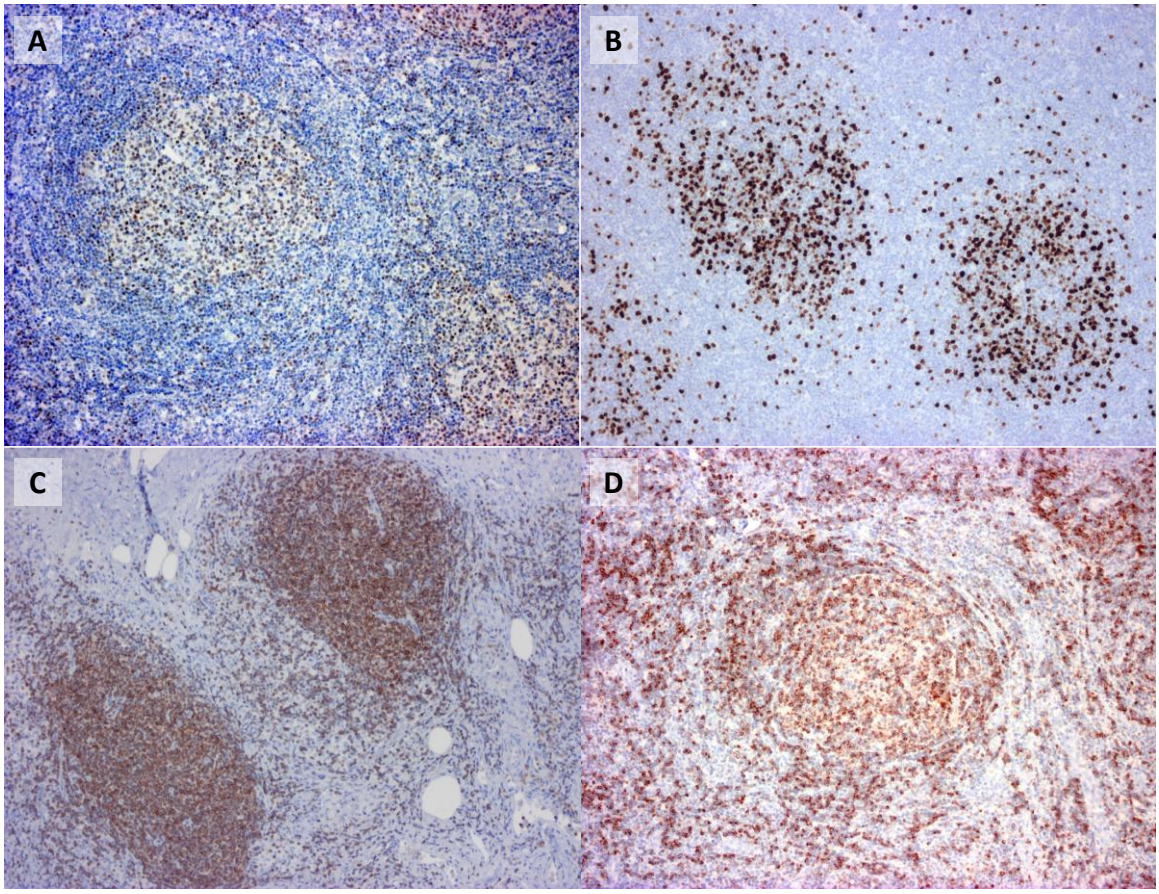

Figure 5: Immunophenotype of follicular lymphoma. A: BCL6 is strongly expressed in the follicles and is frequently downregulated in the interfollicular areas. **B:** Ki67 proliferation marker expressed by the tumour cells. **C and D:** The follicles are uniformly BCL2+.

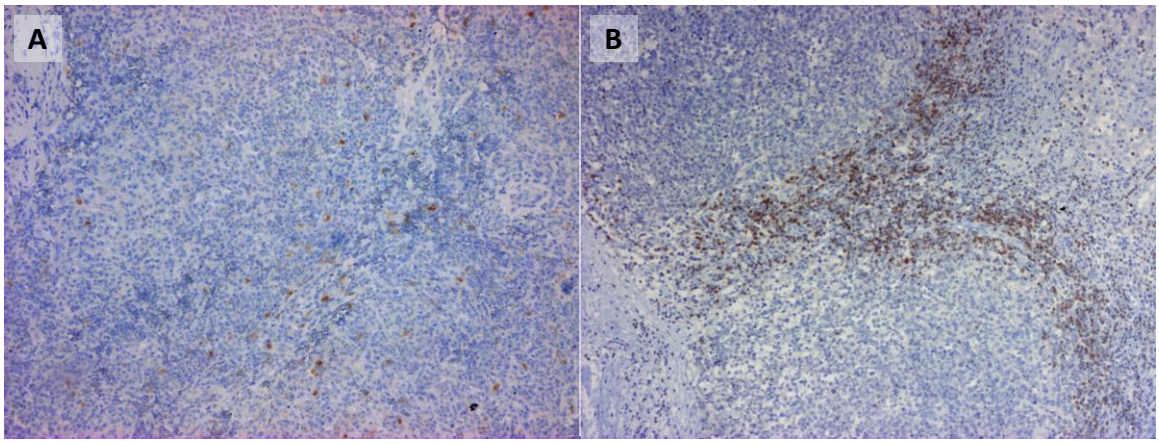

Figure 6: Immunophenotype of follicular lymphoma. A: Some cases, especially FL grade 3B, may lack CD10 expression. **B:** The absence of BCL2 protein in the neoplastic follicles does not exclude FL.

binding site. These mutations enhance the average affinity of the antibodies produced and results in diversification of the *IgV* repertoire [30-32]. Mutations occur mostly at "hotspots" in the DNA known as hypervariable regions, which correspond to the complementarity determining regions (CDR); the sites involved in antigen recognition on the immunoglobulin [30, 33, 34]. Variable region genes undergo further diversification through ongoing somatic mutational activity which generates intraclonal heterogeneity.

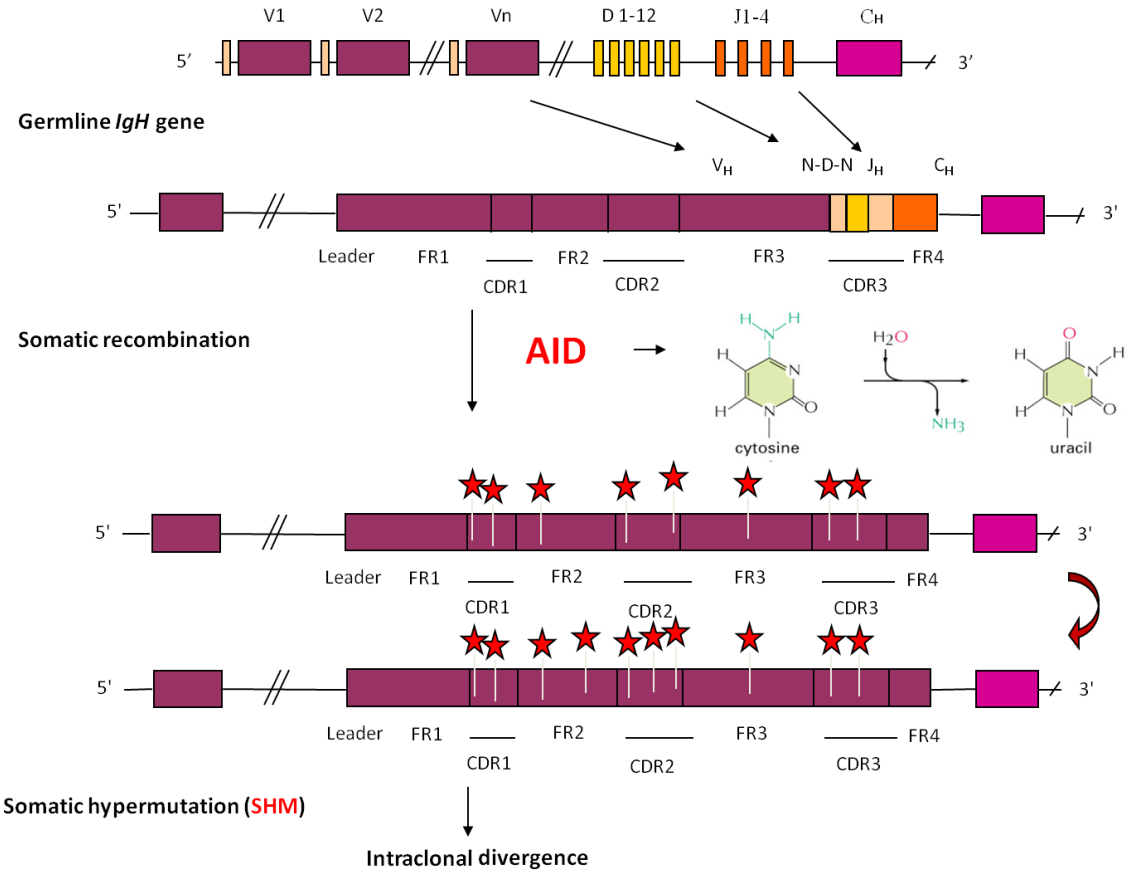

Figure 7: Genetic modifications of *IgH* gene during B-cell development. V(D)J recombination of the germline *IgH* gene occurs in the early bone marrow phase of B-cell development. During the immune response, upon antigen encounter in the GC, B lymphocytes undergo somatic mutations and class switch recombination. These mutations together are responsible for generating high-affinity antibodies of different subclasses that are capable of mediating specific immune responses. Further diversification occurs through ongoing mutational activity which results in intraclonal heterogeneity. SHM and CSR require the AID enzyme activity (CDR: complementarity determining region; FR: framework region).

This continuous diversification of the *IgV* regions is considered to be the hallmark of GC-derived B-cells [5, 29, 30, 35-37]. The mechanism of SHM and CSR involves deamination of cytosine to uracil in DNA by an enzyme called activation-induced (cytidine) deaminase (AID), which is expressed specifically in GC B-cells [38-41]. This feature of the hypermutation mechanism is often responsible for the generation of heavy chain disease, and also for several types of chromosomal translocations of oncogenes next to or into the immunoglobulin loci in human B-cell lymphomas [29]. The ongoing type of somatic mutations can cause genetic instability and the accumulation of additional genetic lesions in the tumour cells can influence the growth, histology, clinical features and therapeutic response of the disease [22, 23, 42, 43].

Approximately 85-90% of FL carry the t(14;18)(q32;q21) chromosomal translocation, juxtaposing the *BCL2* gene with the immunoglobulin heavy chain (*IgH*) gene (Figure 8), resulting in the constitutive expression of BCL2 protein, which induces prolonged cell survival by blocking programmed cell death [44-46]. These events alone are not enough for the transformation of the normal B-cells into FL, since t(14;18) can be detected in more than 50% of healthy individuals, and the incidence of FL is roughly 1 case per 24.000 persons per year in the United States [9]. Moreover, the number of t(14;18)-positive cells is influenced by gender, personal lifestyle and exposure to toxic substances [47]. Furthermore, approximately10-15% of FL do not express BCL2 protein, and approx. 5% do not exhibit the t(14;18) chromosomal translocation either [48]. These latter cases may be therefore misdiagnosed as follicular hyperplasia. If present, the juxtaposed *BCL2* and *IgH* DNA sequences may also provide a marker that can be exploited in the detection of minimal residual disease (MRD). However, the high incidence of the t(14;18) translocation in normal individuals will make it difficult to quantify the frequency of the malignant clone in patients with FL who may similarly carry a background of *Bcl-2/IgH*$^{+}$ cells unrelated to their disease [49].

Alternative translocations of the *BCL2* gene locus with the immunoglobulin light chain genes (*IgL, IgK*) resulting in the translocations t(2;18) or t(18;22) that also lead to an overexpression of BCL2, are very rare in FL. Some cases may also carry the Burkitt lymphoma specific t(8;14) or its variants in addition to t(14;18) [20, 50, 51].

Abnormalities of 3q27 and/or *BCL6* rearrangement are found in 5-15% of FL, most commonly in grade 3B cases [52, 53]. Additionally other gene alterations are also found in 90% of FL and most commonly include the loss of 1p, 6q, 10q, 13q and 17p and gains of chromosomes 1q, 2p, 5, 6p, 7, 8, 12q, X and 18q [54-59].

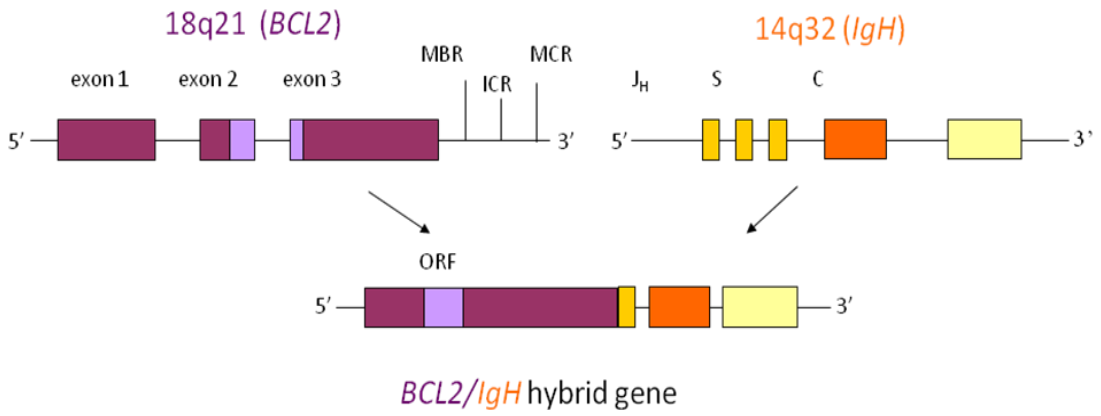

Figure 8: Schematic representation of *BCL2/IgH* gene rearrangement. In t(14;18)(q32;q21) chromosomal translocation the *BCL2* gene is juxtaposed with the *IgH* gene resulting in the constitutive expression of BCL2 protein (MBR: major breakpoint region, MCR: minor cluster region, ICR: intermediate cluster region).

2. 6. The role of BCL2 *gene and BCL2 protein in follicular lymphoma*

BCL2 is a well-known anti-apoptotic protein. Normally, it is expressed in pre B-cells, resting B-cells and certain types of proliferating B-cells. GC B-cells physiologically lack BCL2 expression and undergo apoptosis unless they are selected by specific antigens that drive them into SHM and CSR [60, 61].

The majority of FL overexpress BCL2 protein as a consequence of the t(14;18) translocation, juxtaposing the *BCL2* gene (18q21) with the immunoglobulin heavy chain (*IgH*) gene (14q32), found in approximately 85-90% of cases. The *BCL2* gene was first discovered in FL, and has been considered to be the critical oncogene involved in the pathogenesis of this lymphoma type [45, 62-64]. The majority of FL have a

chromosome 18 translocation at either the major breakpoint region (MBR, 60-70% of cases) or the minor cluster region (MCR, 10-20% of cases) [45, 65-68]. Rare breakpoints are also found in the so called variant cluster region (VCR) or other clusters located between MBR and MCR [69-71]. These translocations result in a fusion gene which contains the full coding region of the *BCL2* gene under the regulation of the *IgH* gene enhancer, and consequently excessive amounts of normal BCL2 protein are produced.

The constitutive overexpression of the BCL2 protein results in the inhibition of apoptosis by blocking the mitochondrial pathway [63, 72], thus leading to an accumulation of inappropriately rescued B-cells with a prolonged life span, which allows the occurrence of additional genetic lesions and consequently leads to tumour formation. This thought to be a critical pathogenic event in the development of FL [44, 46, 60]. Immunohistochemical detection of BCL2 is very helpful in the diagnosis of FL, since the pattern of overexpression in the GC allows distinction from reactive lymph nodes [73]. However, approximately 10-15% of FL do not express BCL2 protein, and a small fraction of FL (approx. 5%) does not exhibit the t(14;18) chromosomal translocation either [48]. These latter cases may be misdiagnosed as follicular hyperplasia.

Because of these so called BCL2-negative FL, lacking BCL2 protein expression and t(14;18), the central dogma regarding the molecular pathogenesis of FL (resistance of tumour cells to apoptosis due to BCL2 overexpression) needs revision. The fact that FL can develop without *BCL2* gene rearrangement and BCL2 protein expression leads to the assumption that there are alternative BCL2-independent pathogenic pathways. It also raises the possibility of other gene product(s) which may provide prosurvival signal(s) which lead to morphologically similar but molecularly distinct FL variants. Thus, the aim of our study was to deepen the knowledge in the pathophysiology of this subset of FL lacking the t(14;18) translocation and BCL2 protein expression.

2. 7. Treatment of follicular lymphoma

Despite substantial improvements in survival, FL is still considered incurable with the available therapies [74]. In many cases treatment may be deferred and a 'watch and wait' approach is appropriate. Studies have consistently shown that there is no survival benefit to treating immediately versus waiting until treatment is necessary [75, 76]. Some of the factors that may indicate the need for treatment according to the GELA Criteria (Groupe d'étude des lymphomes de l'adulte) and NCCN Guidelines (National Comprehensive Cancer Network) include: presence of B symptoms, deterioration of quality of life, bulky tumours >10cm, tumours which are threatening major organs, steady progression, cytopenias or patient preference [77, 78].

No chemotherapy agent or combination regimen prior to the introduction of rituximab (MabThera/Rituxan) had been shown to prolong overall survival [79]. Rituximab is a monoclonal antibody against the CD20 B-cell antigen that has been used successfully, both alone and in combination with standard chemotherapy, in patients whose FL has either recurred or proven resistant to other treatment. In addition, data from several clinical trials have shown that the addition of rituximab also to first-line chemotherapy improves long-term outcomes [80]. The National LymphoCare Study recently reported that 52% of patients with FL in the US receive immunochemotherapy as initial treatment [81]. The three most commonly used regimens in combinations of first- and second-line therapies include R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; 55%), R-CVP (rituximab, cyclophosphamide, vincristine, and prednisone; 23%), and R-Flu (rituximab- and fludarabine-based chemotherapy; 16%). [79]. A recent study published at the 2009 ASH convention shows clearly superior response rates, and progression free survival when combining Bendamustine with rituximab (B-R) compared to the R-CHOP regimen. Lower toxicity is an additional benefit [82]. Another promising option is upfront radioimmunotherapy (Zevalin or Bexxar). The lower toxicity compared to chemotherapy along with impressive results is beginning to make this a very attractive choice. After initial treatment has been completed it is becoming increasingly common to consolidate it by including maintenance rituximab therapy or radioimmunotherapy [24].

The use of autologous stem cell transplantation as a consolidation treatment is restricted to younger, fit patients who do not respond to first line treatment [83]. All forms of consolidation have shown quite conclusively to prolong progression free survival. Still, as conventional therapy for FL is not curative, virtually all patients will at least develop a progressive or recurrent disease. In general, the treatment options for relapsed or refractory disease are similar to those for first-line therapy [24, 84].

2. 8. Prognosis of follicular lymphoma

Although FL is incurable, it usually follows an indolent waxing and waning course. The Follicular Lymphoma International Prognostic Index (FLIPI) includes five independent predictors of inferior survival (based on retrospective analysis): age >60 years, haemoglobin <12 g/dl, serum LDH >normal, Ann Arbor stage III/IV, number of involved nodal areas >4. The presence of 0-1, 2, and ≥3 adverse factors defines low, intermediate, and high-risk disease, respectively with overall median survival of 7 to 9 years. With the use of modern therapy, specifically anti-CD20 monoclonal antibody (rituximab), the outcome has improved [21, 85]. The recent upgrading called FLIPI-2, which includes new parameters, was confirmed in prospective studies to better fit for the risk assessment of immunochemotherapy. The five independent FLIPI-2 predictors of superior survival are: age <60 years, haemoglobin ≥12 g/dl, serum LDH ≤normal, no bone marrow involvement and longest diameter of largest lymph node ≤6 cm [24, 86].

3. The role of genomic instability in tumourgenesis

Tumourgenesis can be viewed as an imbalance between the mechanisms of cell-cycle control and mutation rates within the genes. It is now widely accepted that cancer results from the accumulation of mutations in the genome. There is evidence that most cancers are genetically unstable, which contributes to tumour progression and heterogeneity [87].

Genetic instability can be manifested either at the level of nucleotides or the level of the chromosomes. In a small subset of tumours the instability is observed at the nucleotide level and results in base substitutions, deletions or insertions of a few nucleotides [88]. The main forms are:

- Dysfunction or inactivation of tumour suppressor and DNA mismatch repair (MMR) genes through deletions, point mutations (e.g. $p16^{INK4A}$ gene in mediastinal large B-cell lymphoma [89]) or hypermethylation of promoter regions such as of *hMSH1* (human MutS homologue) gene in CLL [90], which contribute to aberrations in the genome leading to tumourgenesis. Genomic instability may present itself through alterations in the length of short repeat stretches of coding and non-coding DNA, resulting in microsatellite instability (MSI) associated with "mutator" phenotype. In case of FL MSI plays a role in histological transformation of low grade into high grade lymphoma [91].
- A recently described form of genetic instability is the aberrant somatic hypermutation (ASHM), which will be described in more details in chapter 3.1.

In most other cancers, the instability is observed at the chromosome level, resulting in losses and gains of whole chromosomes or large portions thereof [88]. The main forms are:

- Alterations in chromosome number involving losses or gains of whole chromosomes (aneuploidy). Such changes are found in nearly all major human tumour types [87]. In FL the most frequent numerical aberrations are the trisomy of chromosomes 5, 7 and X [59].
- Chromosome translocations which can give rise to gene fusion transcripts with tumourigenic properties. Approximately 85-90% of FL carry the t(14;18) chromosomal translocation, juxtaposing the *BCL2* gene to the immunoglobulin heavy chain (*IgH*) gene, resulting in the constitutive expression of BCL2 protein, which induces prolonged cell survival by blocking programmed cell death.
- Gene amplifications resulting in multiple copies of an 'amplicon' containing 0.5-10 megabases of DNA. An example is the amplification of *n-MYC* that occurs in approx. 30% of advanced neuroblastomas [87].

Telomere length and telomerase activity, important in maintaining chromosomal structure and in regulating a normal cell's lifespan, have been shown to have a function in both suppressing and facilitating malignant transformation [92]. Emerging evidence also suggests that dietary and environmental agents can further modulate the contribution of genetic instability to tumourgenesis [92].

3. 1. Aberrant somatic hypermutation (ASHM)

A recently described form of genetic instability at the nucleotide level is aberrant somatic hypermutation (ASHM) which is considered to be a malfunction of SHM [93]. Somatic hypermutation targets primarily the immunoglobulin variable region genes in germinal centre B-cells. This process introduces single nucleotide substitutions, with rare deletions and duplications, resulting in the production of high-affinity antibodies and allowing affinity-maturation of the humoral immune response [30-32]. It has been also described that at least 3 non-Ig genes of B-cells, including *BCL6* and *FAS/CD95*, acquire somatic mutations during the normal GC reaction, indicating that this mechanism may target more genes than originally suspected [93, 94]. SHM has been shown to malfunction in about 50% of DLBCL [95], in FL [96], in mediastinal large B-cell lymphoma [97], in about 20% of AIDS-related NHL [98], in primary central nervous system DLBCL [99], in primary cutaneous marginal zone B-cell lymphoma [100] and MALT lymphoma [101]. ASHM has also been associated with FL and CLL transformation into higher grade DLBCL [102, 103]. In these tumours, multiple somatic mutations are introduced into the 5' region, including coding sequences of several genes that do not represent physiologic SHM targets. These are the well-known proto-oncogenes *c-MYC*, *RhoH*, *PAX-5* and *PIM1* (Figure 9) [93, 95].

The *c-MYC* gene encodes a transcription factor involved in the control of cell growth, proliferation, differentiation and apoptosis [95]. Tumour-associated mutations of this gene have been observed in endemic Burkitt lymphoma carrying t(8;14) translocations [104, 105]. The majority of mutations are alternatively distributed on

1,5 – ~0,9 kb long fragments in the region downstream to the major P1/P2 (exon 1) or the region downstream to the minor P3 promoter (exon 2) [95].

Another gene frequently targeted by ASHM is the *RhoH*, which encodes a small GTP-binding protein belonging to the *RAS* superfamily. This gene is involved in rare tumour-associated translocations that juxtapose its coding domain to the *Ig* locus. Mutations occur in the 1B exon 3' region including non-coding sequences [95].

PAX-5 encodes a B-cell specific transcription factor essential for B-lineage commitment and differentiation, and it is involved in translocations in ~50% of lymphoplasmocytoid lymphoma. Somatic mutations were identified predominantly around exon 1B, on 1 kb long fragments [95].

The fourth gene targeted by ASHM is *PIM1*, a proto-oncogene identified as a preferential proviral integration site in murine T-cell lymphomas [106] and occasionally involved in DLBCL-associated chromosomal translocations. Mutations are distributed in a region spanning up to 2 kb towards the 3' end from the transcription initiation site, and are clustered in a stretch of ~ 1,2 kb [95].

The mutation pattern introduced by ASHM corresponds to the specific features of the SHM process seen in physiologic targets like *IgV* and *BCL6*:

- mutations are distributed in a region spanning up to 2 kb towards the 3' end from the transcription initiation site,
- predominance of single nucleotide substitutions with occasional deletions or duplications,
- preference for transitions (purine to purine or pyrimidine to pyrimidine) over transversions (purine to pyrimidine or pyrimidine to purine),
- elevated ratio of G+C over A+T substitutions,
- and display a preferential distribution within the RGYW motif (R=A/G, Y=C/T, W=A/T) [107-109].

DOI:10.14753/SE.2012.1744

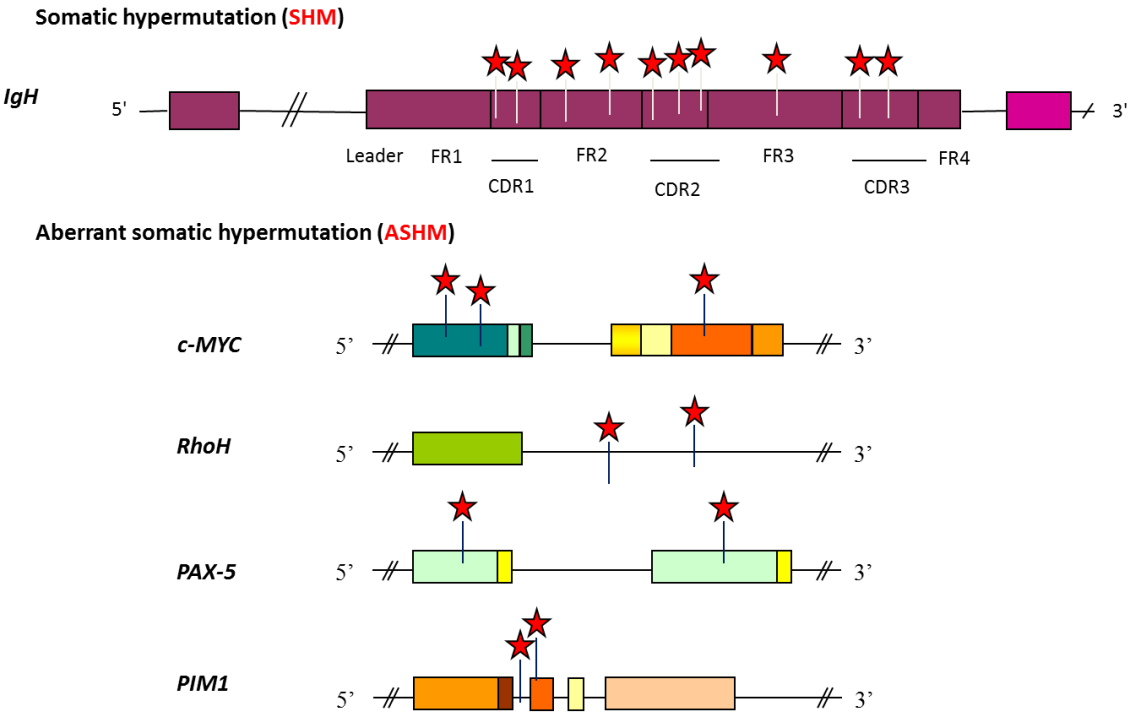

Figure 9: Schematic representation of SHM and ASHM activity. Somatic hypermutation occurs in GC B-cells where many non-random, single-base changes are introduced into the *IgV* region. These mutations result in the production of high-affinity antibodies and allow affinity-maturation of the humoral immune response. The SHM has been shown to malfunction in certain lymphoma types. In these tumours, ASHM introduces several mutations into well-known proto-oncogenes like *c-MYC*, *RhoH*, *PAX-5* and *PIM1*.

3.2. Activation-induced (cytidine) deaminase (AID)

The *AID* gene, encoding for activation-induced (cytidine) deaminase, has been recently identified as an absolute requirement for both SHM and CSR [38, 110]. Absence of *AID* in knockout mice leads to absent CSR and defective SHM, and to severe defect of the humoral immune response [38]. A high proportion of AID is localized in the cytoplasm of CD19+ and CD38+ B-cells. However, AID also has a nuclear localization signal. Both cytoplasmic and nuclear AID levels increase following stimulation of B-cells for CSR. It has been suggested that AID might be sequestered in the cytoplasm of B-cells until required, possibly to limit its mutator activity [111].

Because AID is a close homologue of APOBEC-1, an RNA-dependent cytidine deaminase, it has been proposed that AID may function as a cytidine deaminase which modifies a preexisting mRNA into a new one, by possibly encoding an endonuclease. However, experimental evidence indicated that AID acts directly on single-stranded DNA by converting deoxy-cytidines (dC) to deoxy-uracils (dU) [93, 112, 113]. The deamination that results from AID activity creates a uracil-guanine (U-G) mismatch in the DNA. The generated uracil can be processed through different pathways, resulting in different mutations on selected genes (Figure 10). One way of processing the mismatch is replication without repair. In this situation C-T and G-A transitions appear.

If processed through the base excision repair pathway (BER), the uracil will be cleaved by DNA uracil N-glycosylase (UNG) to generate an abasic site, which will be further cut by apurinic-apyrimidinic endonuclease (APE), resulting in a nick on that strand. The nick can either be repaired with error-free replication or bypassed by error-prone polymerase (EPP) to generate all possible mutations. It also can be recognized by the mismatch repair (MMR) proteins and then excised and replaced with error-prone polymerases that will create additional mutations. If the mutations are present in late S and G2, they can be repaired by homologous recombination [114]. DNA double-strand breaks are obligate intermediates in the CSR and it has been suggested that AID may also be responsible for lesions that lead to translocations involving switch regions. AID has been described to be essential for *c-MYC* translocations to the *Ig* switch region in Burkitt lymphoma [115].

DOI:10.14753/SE.2012.1744

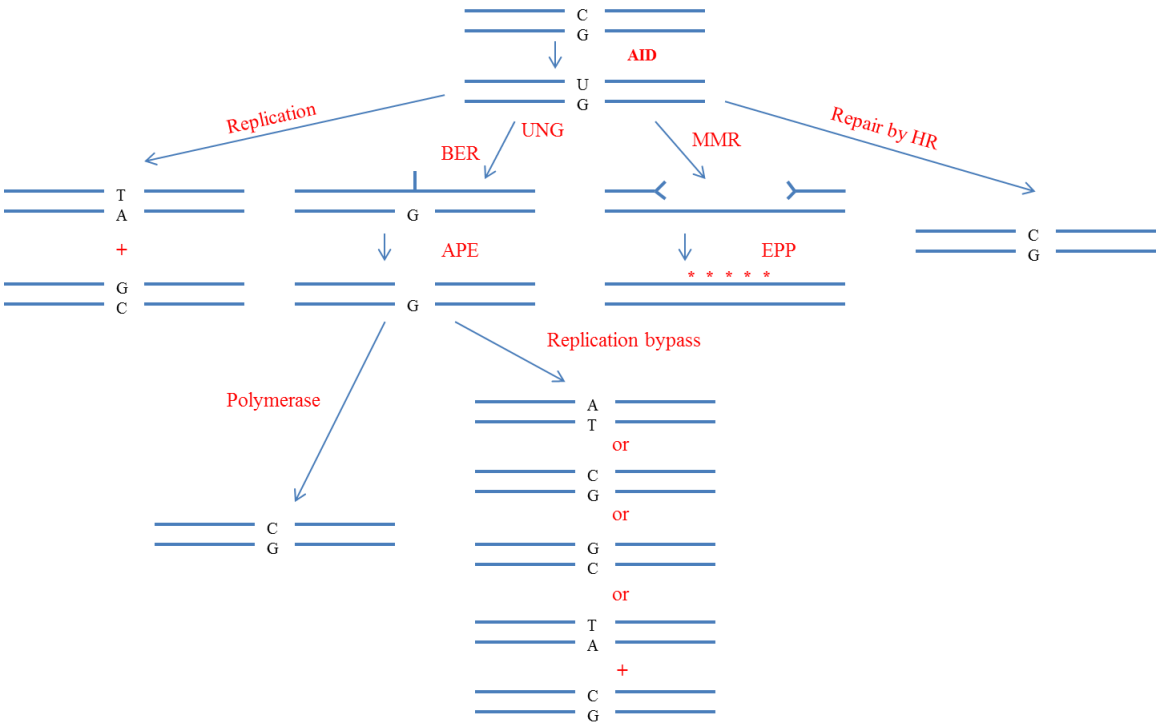

Figure 10: Resolution of U-G mismatch created by AID on DNA. AID deaminates deoxy-cytidine (dC) to deoxy-uracil (dU) to create U-G mismatches. The uracil can be processed through different pathways. One way of processing the mismatch is replication without repair, where C to T mutations appear. If processed through the base excision repair pathway (BER), the uracil will be cleaved by DNA uracil N-glycosylase (UNG) to generate an abasic site, which will be further cut by apurinic-apyrimidinic endonuclease (APE), resulting in a nick on that strand. The nick can either be repaired with error-free replication or bypassed by error-prone polymerase to generate all possible mutations (transitions and transversions). Another way is through the mismatch repair proteins (MMR). The sequence surrounding the mismatched nucleotide is excised and replaced with error-prone polymerases (EPP), which creates additional mutations of the sequence. Finally, it can be repaired by means of homologous recombination (HR) if the mutations are present in late S and G2 when a sister chromatid is available as a template (Figure after Luo, Z. *et al.*, J Allergy Clin Immunol, 2004).

On the whole, we can conclude that AID mediated DNA lesions by direct deamination constitutes only the initial step and the multitude of mutations are actually due to the various mismatch repair attempts. In agreement with these observations, deregulated expression of AID is associated with malignancy.

DOI:10.14753/SE.2012.1744

In healthy B-cell development the expression of AID is strictly regulated and restricted to GC B-cells, which follows that constitutive expression of AID may contribute to NHL formation. This is also supported by previous findings indicating that AID is overexpressed in FL, DLBCL, Burkitt lymphoma, mediastinal large B-cell lymphoma and MALT lymphoma [93, 97, 101, 116-118].

DOI:10.14753/SE.2012.1744

III. OBJECTIVES

Several reports suggest that FL without translocation and expression of *BCL2* gene have distinct morphological, genetic and molecular characteristics that distinguish them from the BCL2-positive FL.

- We characterized the mutational pattern of IgV_H genes to provide further insight into the molecular pathways of lymphomagenesis and to reveal the cellular origin of FL without *BCL2* involvement.
- We analysed whether *c-MYC, PAX-5* and *RhoH* proto-oncogenes are differently affected by aberrant somatic hypermutation (ASHM) in BCL2-negative FL in comparison with BCL2-positive FL.
- We determined the mRNA and protein expression levels of activation-induced (cytidine) deaminase (AID) in BCL2-negative FL compared to BCL2-positive FL.

DOI:10.14753/SE.2012.1744

IV. MATERIALS AND METHODS

1. Tissue samples

Lymph node (LN) biopsies of 18 patients with FL were selected for this study, based on the availability of frozen tissue for molecular analyses and formalin-fixed paraffin-embedded tissue for immunophenotyping and fluorescence *in situ* hybridization (FISH) analysis. Diagnoses were based on histopathology, immunophenotype, FISH and molecular analyses, and cases were classified according to the World Health Organization (WHO) Classification of lymphoid tumours [20]. The clinical, morphological and immunohistochemical data as well as the results of FISH analyses are summarized in Table 1.

The phenotype of lymphoma cells was characterized using the three-step avidin-biotin immunoperoxidase method with mouse anti-human monoclonal antibodies against: CD20 (clone L26), BCL6 (clone PG-B6p), Ki67 (clone MIB1) (all from DAKO, Carpinteria, CA, USA), and CD10 (clone 56C6, Novocastra Laboratories, Newcastle upon Tyne, UK) antigens. Follicular dendritic cells were detected with anti-CD21 (clone 1F8) and anti-CD23 (clone 1B12) (both from Novocastra) antibodies. Three monoclonal mouse anti-human antibodies against different epitopes were used to study BCL2 protein expression: clone 124 (DAKO) was used as a standard, supported by clone c2 (Santa Cruz Biotechnology Inc, Santa Cruz, USA) and clone 6C8 (Pharmingen, Franklin Lakes, USA) to confirm the results. Cases, in which BCL2 protein expression was undetectable with the routinely used antibody as a result of somatic mutations of the *BCL2* gene, but with the use of the latter two antibodies BCL2 protein expression could be confirmed, were excluded from the study.

FISH analysis was accomplished using a commercially available LSI (locus specific indicator) *BCL2* dual-colour break-apart rearrangement probe (18q21), LSI *BCL6* dual-colour break-apart rearrangement probe (3p27) and LSI *IgH/BCL2* dual-colour, dual-fusion translocation probe set (14q32, 18q21) (all from Vysis, Downers

Grove, IL, USA). At least 200 interphase nuclei per probe were evaluated in each reaction. Cases which lacked the t(14;18) translocation, but in which split signals were present involving the *BCL2* gene, were excluded from further analysis.

Peripheral blood samples from 8 healthy individuals and GC cells microdissected from 5 reactive follicles served as controls for this study.

DOI:10.14753/SE.2012.1744

Table 1. Clinical, morphological and immunohistochemical data and results of FISH analysis of patients with and without *BCL2* translocation and expression.

Case No.	Age (Years)	Sex	Grade	Immunohistochemical analysis*						FISH		
				CD20(%)	CD10(%)	CD21/23	BCL2(%)	BCL6(%)	Ki67(%)	t(14;18)	*BCL2* split	*BCL6* split
1	49	male	3A	100	2**	+	-	90	25	-	-	-
2	46	female	3A	100	100	+	-	30	15	-	-	not informative
3	60	male	2	100	100	+	-	100	10	-	-	-
4	44	male	3A	100	70	+	-	70	10	-	-	-
5	49	female	3A	100	5**	+	-	80	15	-	-	-
6	74	male	3A	100	100	+	-	60	10	-	-	-
7	33	female	3A	100	100	+	-	80	25	-	-	40% - 1 split
8	48	female	2	100	100	+	-	80	15	-	-	-
9	65	male	3A	100	100	+	-	85	40	-	-	55%- 3-4 signals
10	73	female	3A	100	70	+	-	60	35	-	-	-
11	71	male	3A	100	100	+	-	40	70	-	-	60% - 1 split
12	57	male	3A	100	100	+	100	100	25	+	ND	-
13	60	female	3A	100	80	+	85	70	40	+	ND	60% - 3 signals
14	33	female	3A	100	100	+	100	100	40	+	ND	ND
15	66	female	3A	100	100	+	50	40	45	+	ND	-
16	78	female	3A	100	80	+	95	70	20	+	ND	ND
17	50	male	2	100	100	+	60	60	40	+	ND	-
18	60	male	2	100	100	+	90	90	30	+	ND	-

ND, not determined.

* CD21 and CD23 positivity was detected on the follicular dendritic cells, while the other markers stained the tumor cells.

Number values indicate the percentage of cells expressing the marker in each sample.

**Cases 1 and 5 were considered CD10 negative.

DOI:10.14753/SE.2012.1744

2. DNA based analyses

Eighteen fresh frozen lymph node samples of cases of FL were used for DNA based analyses. A schematic representation of the workflow is given in Figure 11. In case of IgV_H genes PCR amplicons were first cloned, then the appropriate insert of plasmid DNA was sequenced, while in case of *c-MYC*, *PAX-5* and *RhoH* genes direct sequencing was used.

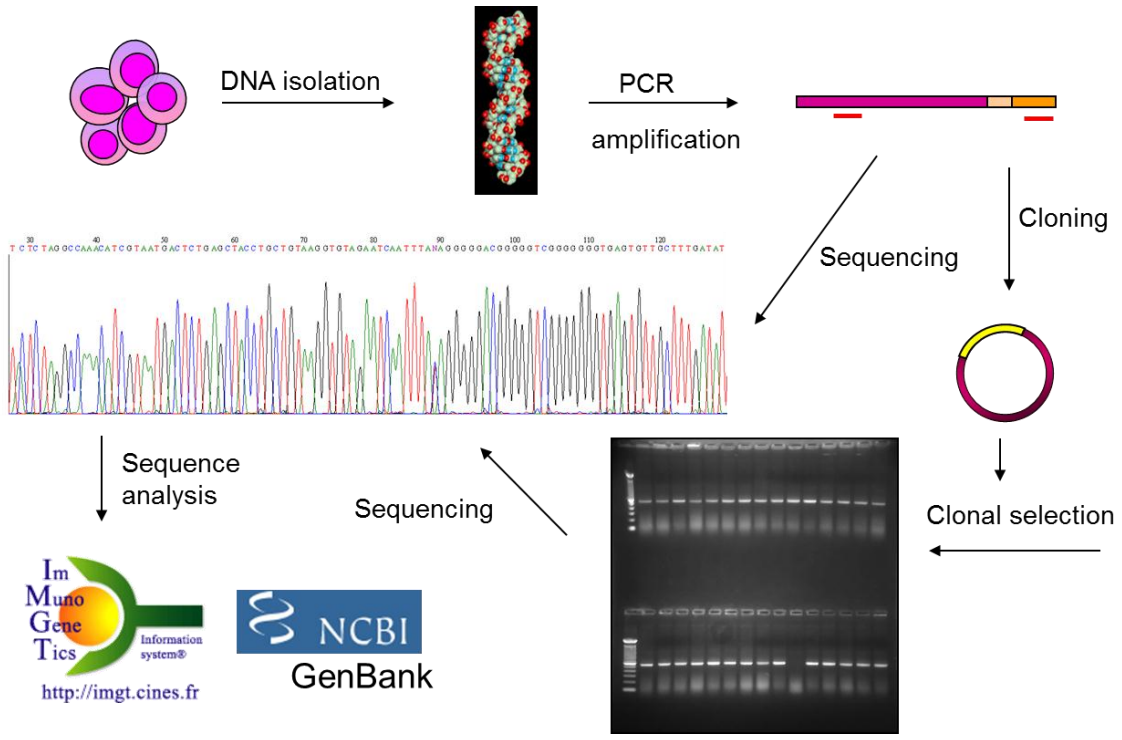

Figure 11: Schematic representation of DNA analyses workflow. DNA isolation was followed by PCR amplification. To characterize the SHM activity on IgV_H genes PCR amplicons were first cloned, then the appropriate insert of plasmid DNA was sequenced, while in case of *c-MYC*, *PAX-5* and *RhoH* genes direct sequencing was used to determine the ASHM mutational activity. The sequence analysis was performed using the IMGT/V-QUEST and the NCBI GenBank databases.

DOI:10.14753/SE.2012.1744

2. 1. DNA isolation

Genomic DNA isolation from tumour tissue specimens was performed according to the standard salting-out procedure [119]. Tumour tissue was suspended in 3 ml nuclei lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA and 5% SDS). The cell lysates were digested overnight at 37 °C with 50 µl protease-K (1 mg protease-K in 1% SDS and 2 mM EDTA). After digestion was complete, 1 ml of saturated NaCl (6M) was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at 2500 rpm for 15 minutes. The precipitated protein pellet was left at the bottom of the tube. The supernatant containing the DNA was transferred to another tube and mixed with 2 volumes of room temperature absolute ethanol. The tubes were inverted several times and the precipitated DNA was washed twice in 80% ethanol. Then the DNA strands were transferred with a sterile needle into a microcentrifuge tube containing 150 µl TE buffer (10 mM Tris-HCl, 2 mM EDTA), allowed to dissolve at 55 °C for 1 hour and measured in a Gene Quant II spectrophotometer (Cambridge, UK) at 260 nm wavelength. The samples were stored at 4 °C until further use.

2. 2. PCR amplification

IgH variable genes are composed of variable (V), diversity (D) and joining (J) gene segments. In humans, there may be a total of 100 to 200 V gene segments, more than 30 D segments and 6 functional J segments. Germline V segments can be grouped into six families (VH1-VH6) based on nucleotide sequence similarity. Members of the same VH family are typically more than 80% homologous, while homology between VH genes from different families is less than 70%. Individual families range in size from one (VH6) to greater than 28 (VH3) members and pseudo as well as functional genes [120]. Tumour DNA samples in our study were amplified by PCR, using sense IgV_H gene family-specific (VH1-VH6) leader primers in conjunction with an antisense consensus J_H

DOI:10.14753/SE.2012.1744

primer in independent reactions [121] (Figure 12 and Table 2). The PCR products obtained were between 300-400 bp (base pair) long.

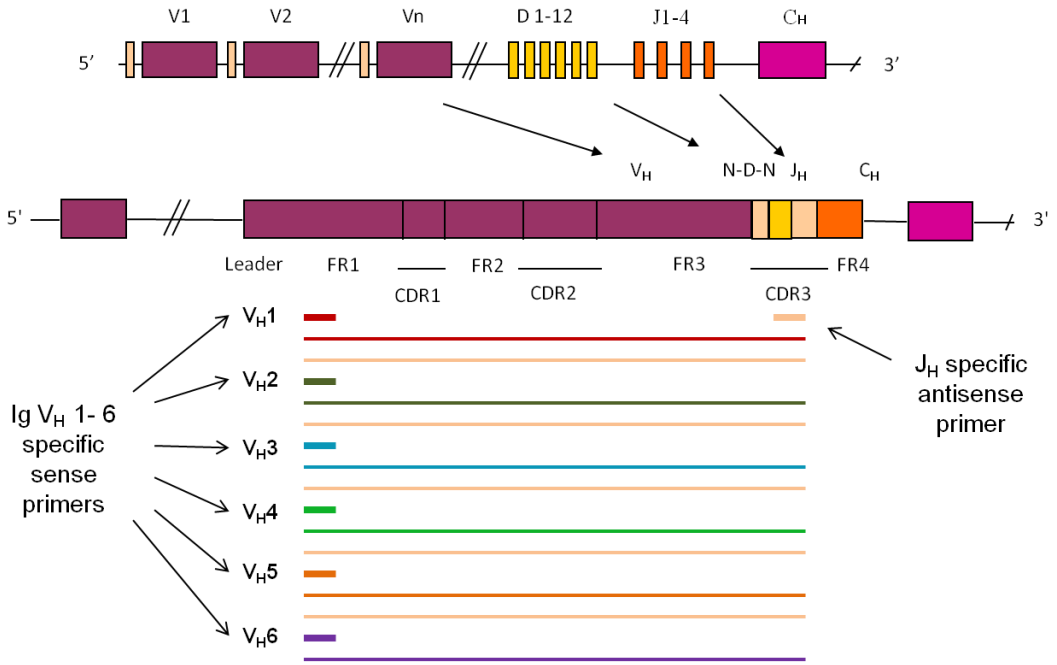

Figure 12: PCR amplification of *IgV$_H$* genes. For the PCR amplification sense *IgV$_H$* gene family-specific (V_H1-V_H6) leader primers (FR1 region) were used in conjunction with an antisense consensus J_H primer (FR4 region). The PCR products obtained were between 300-400 bp long.

Table 2. Melting points and primer sequences used in PCR amplification of *IgV$_H$* genes.

Gene region	***Primer sequence***	***Melting point***
VH1-FR1	5'-GGC CTC AGT GAA GGT CTC CTG CAA G-3'	63 °C
VH2-FR1	5'-GTC TGG TCC TAC GCT GGT GAA ACC C-3'	63 °C
VH3-FR1	5'-CTG GGG GGT CCC TGA GAC TCT CCT G-3'	66 °C
VH4-FR1	5'-CTT CGG AGA CCC TGT CCC TCA CCT G-3'	65 °C
VH5-FR1	5'-CGG GGA GTC TCT GAA GAT CTC CTG T-3'	60 °C
VH6-FR1	5'-TCG CAG ACC CTC TCA CTC ACC TGT G-3'	63 °C
J_H consensus	5'-CTT ACC TGA GGA GAC GGT GAC C-3'	54 °C

DOI:10.14753/SE.2012.1744

PCR reactions were performed in 25 µl final volumes using reagents as shown in Table 3 in a PE 2400 Gene Amp (Perkin-Elmer, USA) thermal cycler. Amplification consisted of 35 cycles using PCR conditions as described in Table 4. Annealing temperature was 58 ^{o}C for VH1 and VH3 and 55 ^{o}C for VH4 for 40 sec with 40 sec denaturation. Annealing temperature for VH2, VH5 and VH6 was 59 ^{o}C for 30 sec with 30 sec denaturation. In all cases two independent reactions were made.

Table 3. Components of PCR reaction used for amplification of IgV_H genes.

Components	***quantity/sample***
PCR Gold buffer (10x)	2.5 µl
$MgCl_2$ (25 mM)	1.5 µl
dNTP mix (2 mM)	10 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
AmpliTaq Gold polimerase (0.75U/µl)	0.15 µl
DNA template	100 ng
Distilled water	up to 25 µl

Table 4. PCR conditions for IgV_H gene amplification.

Step	***Temperature***	***Time***	***Cycles***
Initial denaturation	95 ^{o}C	10 min	-
Denaturation	95 ^{o}C	30/40 sec	35
Annealing	55/58/59 ^{o}C	30/40 sec	
Extension	72 ^{o}C	50 sec	
Final extension	72 ^{o}C	10 min	-

Mutational analysis of *c-MYC*, *PAX-5* and *RhoH* genes was performed on selected regions previously shown to contain more than 90% of the mutations [95]. Primer sequences used are shown in Table 5. PCR amplification was performed in two independent reactions using the Phusion™ High-Fidelity DNA Polymerase system (Finnzymes, Finland) containing a low-error DNA polymerase. c-*MYC* (exon1 and exon 2), *PAX-5* and *RhoH* genes were PCR-amplified as described previously [98]. PCR reactions were performed in 25 µl final volumes using reagents as shown in Table 6 in a PE 2400 Gene Amp (Perkin-Elmer, USA) thermal cycler. Amplification consisted of 35 cycles using PCR conditions as described in Table 7.

Table 5. Melting points and primer sequences used in PCR amplification of *c-MYC*, *PAX-5* and *RhoH* genes.

Gene region	***Primer sequence***	***Melting point***
c-MYC exon-1	5´-CAC CGG CCC TTT ATA ATG CG-3´ 5´-CGA TTC CAG GAG AAT CGG AC-3´	62 °C 59 °C
c-MYC exon-2	5´-CTT TGT GTG CCC CGC TCC AG-3´ 5´-GCG CTC AGA TCC TGC AGG TA-3´	66 °C 61 °C
PAX-5	5´-CCC AGA GAC TCG GAG AAG CA-3´ 5´-AAG AGC TGA AAT GTC GCC GCC G-3´	60 °C 64 °C
RhoH	5´-CCT TAA AAG TAT TTC TTT GGT GTC-3´ 5´-AAC TCT TCA AGC CTG TGC TG-3´	55 °C 55 °C

Table 6. Components of PCR reaction for *c-MYC*, *PAX-5* and *RhoH* genes.

Components	***quantity/sample***
High fidelity buffer (10x)	2 µl
dNTP mix (2 mM)	2 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Triple Master polimerase (5U/µl)	2 µl
DNA template	100 ng
Distilled water	up to 25 µl

DOI:10.14753/SE.2012.1744

In case of *c-MYC* exon 1, *PAX-5* and *RhoH* genes the annealing temperature was 58 ^{o}C, while in case of *c-MYC* exon 2 the temperature was 68 ^{o}C based on the melting point of the primers used. Sizes of PCR products obtained were 1300 bp for *c-MYC* exon 1, 580 bp for *c-MYC* exon 2, 859 bp for *PAX-5* and 844 bp for *RhoH*.

Table 7. PCR conditions for *c-MYC, PAX-5* and *RhoH* gene amplification.

Step	*Temperature*	*Time*	*Cycles*
Initial denaturation	94 ^{o}C	2 min	-
Denaturation	94 ^{o}C	30 sec	35
Annealing	58/68 ^{o}C	45 sec	
Extension	72 ^{o}C	50 sec	
Final extension	72 ^{o}C	7 min	-

2. 3. Separation, detection and purification of PCR products

Products were electrophoresed (130 V for 45 min) through 2% agarose gels (2g agarose, 100 ml 1x TAE buffer) in 1x TAE buffer (10x TAE buffer: 0.4M Tris, 0.2M acetic-acid, 0.01M EDTA) containing ethidium bromide (1 µg/ml). The migrational pattern was visualized by exposure to UV light and photographed using the Kodak 4400 MM (Eastman Kodak Company, Rochester, NY, USA) gel documentation system.

After PCR, amplified DNA must be separated from excess reaction components that can interfere with cloning or sequencing. Products were purified using the Montage PCR Filter Device (Millipore, Billerica, USA) according to the manufacturer's recommendations. This process concentrates amplified DNA and removes primers and unincorporated dNTPs.

DOI:10.14753/SE.2012.1744

2. 4. Cloning of $\mathbf{IgV_H}$ *genes*

In order to study the presence of ongoing mutational activity and intraclonal heterogeneity IgV_H amplicons were cloned using the pCR 2.1-TOPO TA Cloning Kit (Invitrogen Corporation, San Diego, CA, USA). PCR products were ligated in pCR 2.1-TOPO Vector (Figure 13), then the recombinant vector was transformed using One Shot® Top10 *E. coli* Chemically Competent cells according to the manufacturer's recommendations.

The pCR 2.1-TOPO TA Cloning method is adequate for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxy-adenosine d(A) to the 3' end of PCR products. The vector in this kit is supplied linearized with single 3'-deoxy-thymidine d(T) overhangs. This allows PCR inserts to ligate efficiently with the vector.

For each transformation LB agar plates (10g Trypton, 10g NaCl, 5g Yeast Extract, 10g Bacto agar for 1000 ml deionized water) containing 50 µg/ml kanamycin and 40 mg/ml X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase) were used. Because the vector was containing a kanamycin resistance gene, only cells containing the plasmid can survive. After 24-48 hours incubation period at 37 °C, blue and white colonies were seen on the plate. If the ligation was successful, the bacterial colony was white; if not, the colony was blue. The blue/white colony screening is based on the fact that *E. coli* strains are having a β-galactosidase (*lac*Z) gene. When a sequence is inserted into this region, the reading frame is disrupted, and the cell loses its β-galactosidase activity, and therefore these colonies remain white. Any colony containing the plasmid without the insert, and therefore the functioning β-galactosidase gene, turns blue as a result of the enzyme activity detected with X-gal degradation.

DOI:10.14753/SE.2012.1744

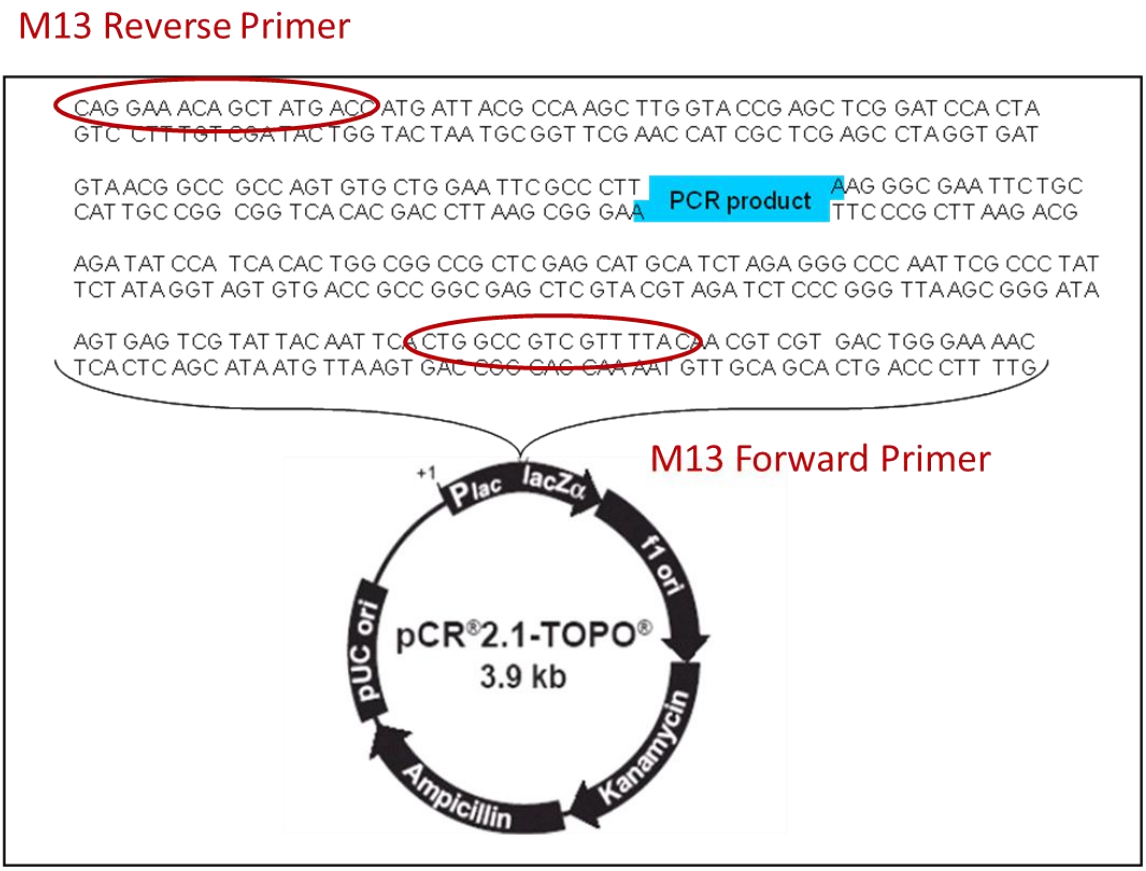

Figure 13: Schematic representation of pCR®2.1-TOPO® Vector. The pCR®2.1-TOPO® Vector is a linearized vector. The vector contains a kanamycin resistance gene, consequently only cells containing the plasmid can survive on kanamycin containing media. If the ligation was successful, the bacterial colony is white because cells lose their β-galactosidase activity by disrupting the *lac*Z gene; if not, the colony turns blue. Clonal selection is made by selecting the white colonies and PCR amplifying them with M13 Forward and M13 Reverse primers (Original source of the figure is the TOPO TA Cloning User Manual, Invitrogen).

2. 5. Clonal selection

In each sample 30 independent bacterial isolates were analysed. White colonies were selected for PCR amplification using the M13 forward and M13 reverse primers:

M13 Forward	5'-CTG GCC GTC GTT TTA C-3'
M13 Reverse	5'-CAG GAA ACA GCT ATG AC-3'

PCR conditions were the same as for the VH1-VH6 amplification (Table 3 and Table 4).

2. 6. Sequencing

Automated DNA sequencing relies on the chain termination method by Sanger. This method is based on the fact that a replicating DNA strand that has incorporated a modified synthetic nucleotide, known as dideoxy-nucleotide (ddNTP), cannot elongate beyond that point. Unlike a "normal" deoxy-nucleotide (dNTP), which lacks a hydroxyl group on its 2' carbon, a dideoxy-nucleotide lacks an additionally hydroxyl group on its 3' carbon. 3' hydroxyl groups are mandatory for the phosphodiester linkage which forms between two consecutive nucleotides. Thus, dideoxy-nucleotides terminate elongation during DNA replication and differently sized fragments are made. Dye-terminator sequencing utilizes labelling of the chain terminator ddNTPs (Figure 14). Each of the four dideoxy-nucleotide chain terminator (ddATP, ddCTP, ddGTP and ddTTP) is labelled with fluorescent dye, each of which emits light at different wavelength when excited by an argon ion laser. DNA sequencer machines carry out capillary electrophoresis for size separation, detection and recording of dye fluorescence, and data output as fluorescent peak trace chromatograms.

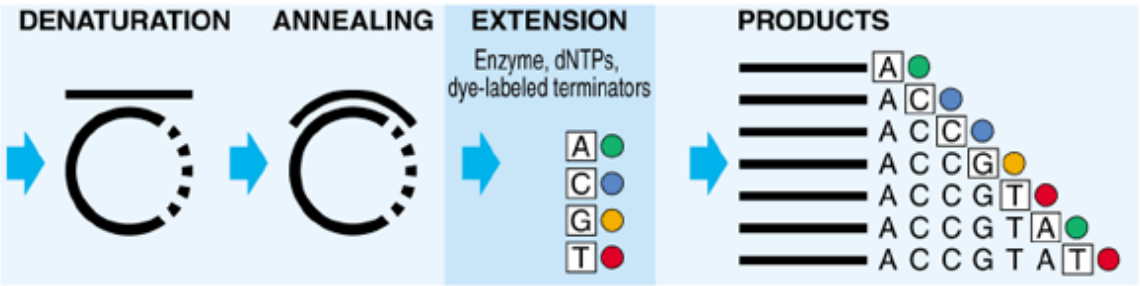

Figure 14: Schematic representation of one cycle of dye terminator cycle sequencing. With dye terminator labelling each of the four ddNTPs is tagged with a different fluorescent dye. The growing chain is simultaneously terminated and labelled with the dye that corresponds to that base. All four colours and therefore all four bases can be detected in a single capillary injection (Original source of the figure is the Applied Biosystems teaching material, www.appliedbiosystems.com).

After IgV_H cloning and transformation of competent cells, colonies found to contain the appropriate insert of plasmid DNA were sequenced. In all samples 20 sequences from independent bacterial isolates were analysed. Direct sequencing of purified amplicons of *c-MYC* exon 1 and exon 2, *PAX-5* and *RhoH* genes was carried out using the same procedure. PCR product sequences were determined in both directions using the Big Dye Terminator 3.1 (Applied Biosystems) kit. The PCR components and conditions are summarized in Table 8 and Table 9. The reactions were carried out by the MiniCycler™ (MJ Research Inc., USA) thermal cycler.

Table 8. Components of the cycle sequencing reaction.

Components	*quantity/sample*
BigDye Terminator	1 µl
BigDye buffer (10x)	1 µl
Primer (10 µM)	1 µl
PCR sample	2 µl

Table 9. PCR conditions for the sequencing reaction.

Step	*Temperature*	*Time*	*Cycles*
Initial denaturation	95 °C	2 min	-
Denaturation	95 °C	30 sec	29
Annealing	50 °C	15 sec	
Extension	60 °C	4 min	

After the sequencing reaction, it is important to remove unincorporated dye terminators and salts that may compete for capillary electrophoretic injection. Unincorporated terminators can co-migrate with the sequencing template, resulting in basecalling errors, and excess salt translates to poor signal-to-noise ratios. Clean-up of sequencing reactions was done by NucleoSeq® Dye-terminator removal according to the manufacturer's recommendations.

Capillary electrophoresis was carried out with an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Eelectrophoresis data were analysed by the BioEdit (Isis Pharmaceuticals, USA) Sequence Alignment Editor.

2.7. Sequence analysis

The corresponding germline IgV_H gene sequences were determined, using the IMGT/V-QUEST (International ImMunoGeneTics Information System, http://imgt.cines.fr) and the NCBI GenBank databases.

The first exposure to antigen results in recruitment of B-cell clones that bind antigen as a result of their combinatorial and junctional specificity of their unmutated surface receptors. Subsequent exposures to antigen lead to accumulation of somatic point mutations in the antibody V segments and antigen selection of the high-affinity mutated B-cell clones. Serial rounds of mutation and selection eventually result in restriction of the response to those B-cells with the best 'fit' for antigen. The variable (V) genes of antigen-selected antibodies are known to exhibit a higher frequency of amino acid replacement mutations in the sequences encoding the antigen-contacting complementarity-determining regions (CDRs) than in those encoding the 'structural' framework regions (FRs). Consequently the CDR sequences have a higher R:S mutation ratio, whereas FR sequences display a lower inherent R:S mutation ratio values than would be expected for a random sequence [122, 123].

In the absence of negative or positive selective pressure on the gene product, a random mutational process would cause an even distribution of nucleotide changes yielding amino acid replacement (R or replacement mutations) and nucleotide changes not yielding amino acid replacements (S or silent mutations) throughout the coding sequence [122].

The binomial distribution model proposed by Chang and Casali (1994) [122] was used to determine whether the probability of the excess of R mutations in the CDR- and the scarcity of S mutations in the FR regions resulted from chance alone or were the

consequence of antigenic selection. The results were then compared with results obtained by the multinomial distribution model suggested by Lossos *et al.* (2000), using the JAVA applet available at http://www-stat.stanford.edu/immunoglobin [123].

The obtained sequences in case of *c-MYC* exon 1 and exon 2, *PAX-5* and *RhoH* genes were compared to the corresponding germline gene sequences available in the NCBI GenBank database. The accession numbers have already been published previously: *c-MYC:* X00346, *PAX-5:* AF386791, *RhoH:* AF386789 [95]. Previously reported polymorphisms and mutations appearing more than once in separate cases, thus considered as polymorphic variants, were excluded from further analyses. Mutation frequency was calculated on the entire region analysed (n – total number of mutations found) and mutated cases only, taken into account 2 alleles (N).

$$\text{Mutation frequency / 100 bp} = \frac{n}{2\,N} * 100$$

3. RNA based analyses

Analysis of *AID* expression was carried out in fresh frozen lymph nodes of eight cases of BCL2-negative FL and seven cases of BCL2-positive FL. Mononuclear cells from peripheral blood (PB) of eight healthy volunteers were used as negative controls and GC cells microdissected from five reactive lymph nodes, using a Leica laser microdissection system (Leica Microsystems, Wetzlar, Germany), were used as positive controls. A schematic representation of the workflow is given in Figure 15.

DOI:10.14753/SE.2012.1744

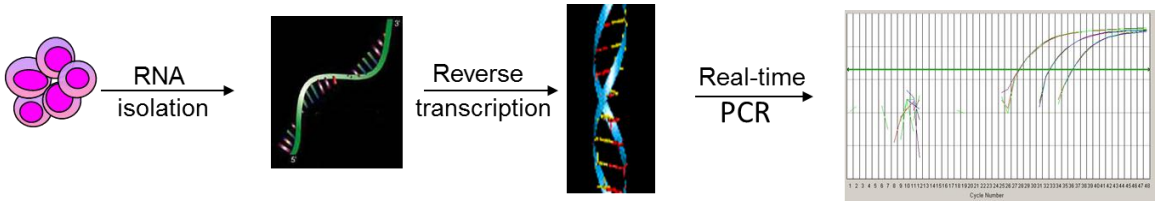

Figure 15: Schematic representation of RNA analyses workflow. RNA was isolated from GC cells, 8 BCL2-negative FL, 7 BCL2-positive FL and 8 peripheral blood samples. Following reverse transcription *AID* expression levels were detected by quantitative real-time PCR assay.

3. 1. Laser microdissection

GC cells microdissected from five reactive lymph nodes, using a Leica laser microdissection system (Leica Microsystems, Wetzlar, Germany) were used as positive controls. 8 μm thick sections were made from frozen tissue on special laser microdissection slides and stained with toluidine-blue (Sigma, St. Louis, USA). Microdissected cells were collected in Trizol® (Invitrogen, Carlsbad, CA, USA) reagent. In order to avoid RNA degradation all materials were treated with RNA later (Qiagen, USA) reagent.

3. 2. RNA isolation

Total RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer.

In cases of PB lymphocytes were separated using Histopaque® (Sigma-Aldrich, St. Louis, USA) and density gradient centrifugation. 2 ml of EDTA anticoagulated blood was applied on 2 ml Histopaque, and then centrifuged on 400g, at 25 ^{o}C for 30 min. The separated mononuclear fraction was transferred to a new tube and homogenized with 5 ml DEPC-PBS (diethyl-pyrocarbonate, phosphate buffer saline), then centrifuged again on 250g, at 25 ^{o}C for 10 min. The supernatant was discarded and the purified mononuclear cells were resuspended in 1 ml Trizol reagent. In cases of FL 30 μm frozen tissue was cut from each sample and homogenized in 1 ml Trizol. The homogenized

samples were incubated for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex. Then 200 ml of chloroform was added and vortexed for 30 sec. After 3 min of incubation at room temperature samples were centrifuged on 10.000g, at 4 ^{o}C for 15 min. The upper aqueous phase containing the RNA was removed to a new sterile tube and homogenized with 0.5 ml isopropanol. After 10 minutes of incubation at room temperature tubes were centrifuged on 10.000g, at 4 ^{o}C for 10 min. The supernatant was removed and the RNA pellet was washed with 1 ml 75% ethanol and then centrifuged at 10.000g, at 4 ^{o}C for 10 min. The wash was discarded and the RNA pellet was air dried, then resuspended in 50 µl DEPC-treated (diethyl-pyrocarbonate) water. The samples were then incubated in a water-bath at 37 ^{o}C for 10 min. The RNA concentration was measured by spectrophotometer (Gene Quant II, Cambridge, UK) at 260 nm wave length. The samples were stored at -70 °C until further use.

3. 3. Reverse transcription (RT)

RNA (2.5 µg) was reverse transcribed to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The reaction components are shown in Table 10.

Table 10. Components of the reverse transcription reaction.

Components	*quantity/sample*
Reverse Transcription Buffer (10x)	10 µl
dNTPs (25x)	4 µl
Random Primers (10x)	10 µl
MultiScribe Reverse Transcriptase (50U/ µl)	5 µl
RNA template	2.5 µg
Nuclease-free water	up to 100 µl

DOI:10.14753/SE.2012.1744

The reverse transcription was made in MiniCycler™ (MJ Research Inc., USA) thermal cycler with the following temperature profile: 10 min incubation at 25 °C, then synthesis at 37 °C for 120 min. The cDNA was stored at -20 °C till further use.

3. 4. Quantitative real-time polymerase chain reaction (RT-PCR) analysis of AID mRNA expression

Analysis of *AID* expression was carried out on fresh frozen lymph node specimens in eight cases of BCL2-negative FL and seven cases of BCL2-positive FL, PB of eight healthy volunteers were used as negative controls and GC cells microdissected from five reactive lymph nodes were used as positive controls. For amplification of AID mRNA a TaqMan® based Gene Expression Assay (Applied Biosystems) was used. The *AID* primers chosen amplified the wild-type and several splice variants of *AID*. In order to normalize the *AID* values, *β-actin* was amplified using a pre-developed TaqMan® Control Reagent (Applied Biosystems).

The schematic representation of the TaqMan® probe chemistry mechanism is shown in Figure 16. TaqMan® probes, also called Double-Dye Oligonucleotides, are 20-30 bp long sequences. A fluorophore is attached to the 5' end of the probe, called reporter (R) and a quencher (Q) to the 3' end. The fluorophore is excited by laser and passes its energy, via FRET (Fluorescence Resonance Energy Transfer) to the quencher. The TaqMan probe binds to the amplicon during each annealing step of the PCR. When the *Taq* polymerase extends from the primer which is bound to the amplicon, it displaces the 5' end of the probe, which is then degraded by the 5'-3' exonuclease activity of the *Taq* polymerase. Cleavage continues until the remaining probe melts off the amplicon. This process releases the fluorophore and quencher into solution, spatially separating them (compared to when they were held together by the probe). This leads to an irreversible increase in fluorescence of the reporter-dye. The increasing fluorescence emission of the reporter is direct proportional with the increasing quantity of the synthesized PCR product, and in the long run with the RNA quantity of the sample.

DOI:10.14753/SE.2012.1744

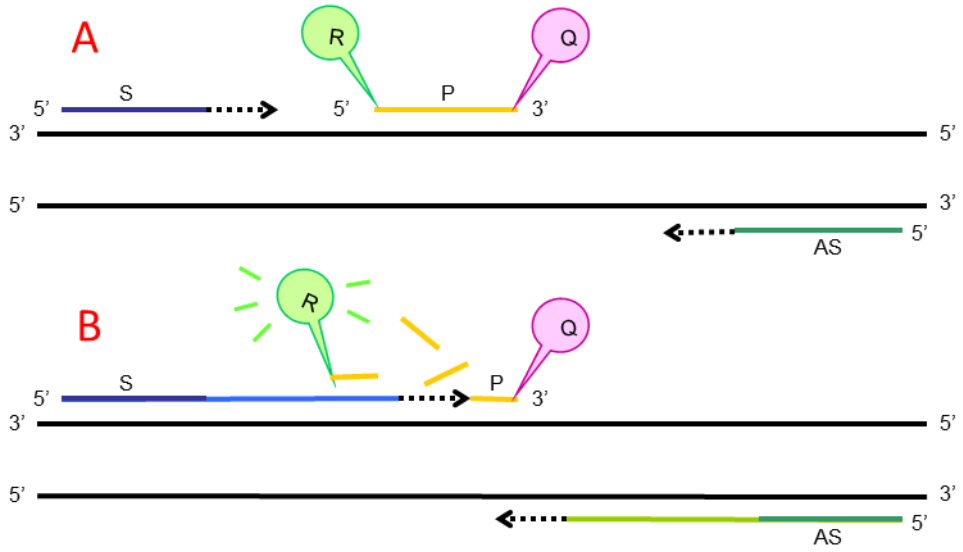

Figure 16: TaqMan® probe chemistry mechanism. The TaqMan® principle relies on the 5´–3´ exonuclease activity of Taq polymerase to cleave a dual-labelled probe during hybridization to the complementary target sequence and fluorophore-based detection. The resulting fluorescence signal permits quantitative measurements of the accumulation of the product during the exponential stages of the PCR. (R: reporter, Q: quencher, S: sense primer, AS: antisense primer, P: TaqMan® probe)

All samples were run in triplicate, in a 20 µl reaction volume that contained 100 ng of cDNA (Table 11).

Table 11. Components of quantitative RT-PCR reaction.

Components	***quantity/sample***
TaqMan Universal PCR Master Mix (2x)	10 µl
Assays-on-Demand (AID/ β-actin) (20x)	1 µl
cDNA template	100 ng
Nuclease-free water	up to 20 µl

DOI:10.14753/SE.2012.1744

The quantitative real-time PCR assay was performed on an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) based on conditions shown in Table 12.

Table 12. Thermal profile of quantitative RT-PCR.

Step	*Temperature*	*Time*	*Cycles*
Activation	50 °C	2 min	
Initial denaturation	94 °C	10 min	-
Denaturation	94 °C	15 sec	50
Annealing-Extension	60 °C	1 min	

Sequence Detection Software version 1.3 (Applied Biosystems) was used to analyse the data. Results were obtained as threshold cycle (C_T) values. C_T represents the cycle number at which fluorescence passes a fixed threshold in the exponential phase of the reaction. The relative quantitation of gene expression was performed by the comparative C_T method ($\Delta\Delta C_T$) (Figure 17). To obtain accurate relative quantitation of an mRNA target, it is recommended to also evaluate the expression level of an endogenous control. The levels of AID expression in the samples were normalized with *β-actin* expression. All values represent the average of three measurements made in case of each sample: $C_{T\ (\text{AID-TARGET})}$, $C_{T\ (\text{AID-CONTROL})}$, $C_{T\ (\beta\text{-ACTIN-TARGET})}$, $C_{T\ (\beta\text{-ACTIN-CONTROL})}$. The calculations were made using the following formulas:

$$\Delta C_{T\ (\text{AID})} = \mathbf{C_{T\ (AID\text{-}TARGET)} - C_{T\ (AID\text{-}CONTROL)}}$$

$$\Delta C_{T\ (\beta\text{-ACTIN})} = \mathbf{C_{T\ (\beta\text{-}ACTIN\text{-}TARGET)} - C_{T\ (\beta\text{-}ACTIN\text{-}CONTROL)}}$$

$$\Delta\Delta C_T = \Delta C_{T\ (\text{AID})} - \Delta C_{T\ (\beta\text{-ACTIN})}$$

$$\textit{Relative expression (AID/}\beta\text{-ACTIN)} = 2^{-\Delta\Delta C_T}$$

The expression values represent the fold changes in the target gene (AID) compared to *β-actin* expression and relative to the expression at time zero (represents the 1x expression of the target gene normalized to *β-actin*).

DOI:10.14753/SE.2012.1744

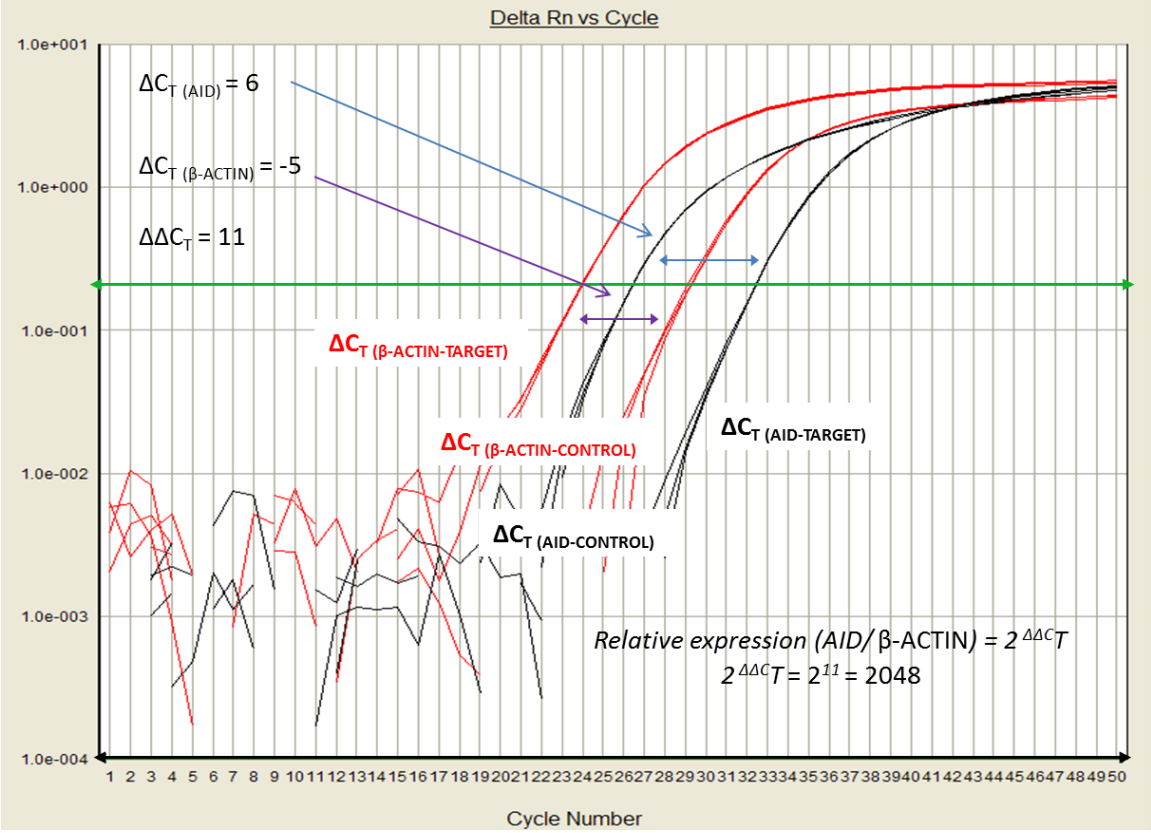

Figure 17: Schematic representation of relative quantitation of gene expression performed by the comparative C_T method ($\Delta\Delta C_T$). The plot shows the relative fluorescence of the samples versus cycle number. Results were obtained as threshold cycle (C_T) values. C_T represents the cycle number at which fluorescence passes a fixed threshold in the exponential phase of the reaction (green horizontal line). All samples were run in triplicate and average values were used. The levels of AID expression in the samples were normalized with *β-actin* expression as endogenous control. The expression values represent the fold changes in the target gene (AID) compared to *β-actin* expression. In the represented case the AID expression in the examined sample was 2048 fold higher than in the control case.

4. Protein based analysis

AID antibodies were used to detect the AID protein level in all 18 FL cases on formalin-fixed paraffin-embedded tissue samples. The 5 µm thick sections were dewaxed in xylene and rehydrated via a series of graded ethanol, finally washed with distilled water. In order to block the endogenous peroxidase activity slides were immersed in 1%

H_2O_2 / methanol for 20 min. Then all samples were submitted to heat-induced epitope retrieval in an AVAIR electric pressure cooker (900 W) in TRS buffer (0.1M, pH=6.1, DAKO) for 18 minutes. For the immunohistochemistry reaction the NovoLink™ (Novocastra) biotin-free, polymer-based detection system was used. The polymer technology uses compact HRP (horseradish peroxidase) secondary antibody conjugates linked to a polymer backbone providing high sensitivity without the risk of non-specific background e.g. from endogenous biotin.

As a first step slides were incubated with the "Protein Block" at room temperature for 10 min in order to avoid the non-specific antibody labelling. After being washed in TBS for 2x5 min, slides were incubated with the primary anti-AID mouse monoclonal antibody (1:100, Ab clone 7E7, Cell Signaling Technology Inc., Massachusetts, USA) at 4 ^{o}C overnight. The next day the samples were washed in TBS for 2x5 min and then incubated with the "Post Primary Block" at room temperature for 30 min. After another step of TBS washing for 2x5 min the slides were incubated with the NovoLink Polymer for 30 min. The immune reactions were visualized with the H_2O_2/DAB substrate/chromogen kit. After rinsing with distilled water the slides were counterstained with haematoxylin, dehydrated in ethanol, cleared in xylene and mounted. The Mirax Scan (Zeiss, Göttingen, Germany and 3DHistech, Budapest, Hungary) was used to digitalize the slides, then the Mirax Viewer software to analyse the reaction and for photo documentation.

5. Ethical considerations

The study was approved by the local Institutional Review Board and was conducted in accordance with the Helsinki Declaration. All patients provided informed consent to the analysis of their data.

DOI:10.14753/SE.2012.1744

V. RESULTS

1. Sequence analysis of IgV_H genes

PCR analysis resulted in amplification in 13 cases; in 5 samples (cases 3, 4, 8, 13 and 18) IgV_H gene mutations were not analysed because of unsuccessful amplification. Steps of data analysis are shown in Figures 18, 19, 20 A and 20 B. The closest germline V_H gene, as well as the sequence homology and mutational frequency of IgV_H genes are detailed in Table 13. The presented sequences were part of the VH3 and VH4 gene families based on nucleotide sequence similarity. Sequence homology ranged between 71.37% - 97.58%.

IgV_H sequence analysis of FL lacking *BCL2* translocation and expression revealed ongoing somatic mutations generating intraclonal heterogeneity of the tumour clones. The average mutational frequency within the IgV_H region of FL cases without *BCL2* gene rearrangement and expression was 12.76% (range 2.41% to 28.62%) which is similar to the average 11.9% frequency (range 9.79% to 16.12%) found in FL harbouring the t(14;18) translocation.

To determine whether the tumour cells of FL had been under pressure for antigen selection, we analysed the somatic mutations of all IgV_H sequences amplified. In all analysed sequences the binomial distribution model revealed more replacement (R) mutations in the complementary determining regions (CDR), and in most cases fewer replacement mutations in the framework region (FR) than could be expected due to chance alone, with significant ($p<0.05$) clustering. The results obtained by the multinomial model revealed statistically significant *p* values, except in one case (case no. 11). The pattern and distribution of these mutations were highly consistent with antigen selection.

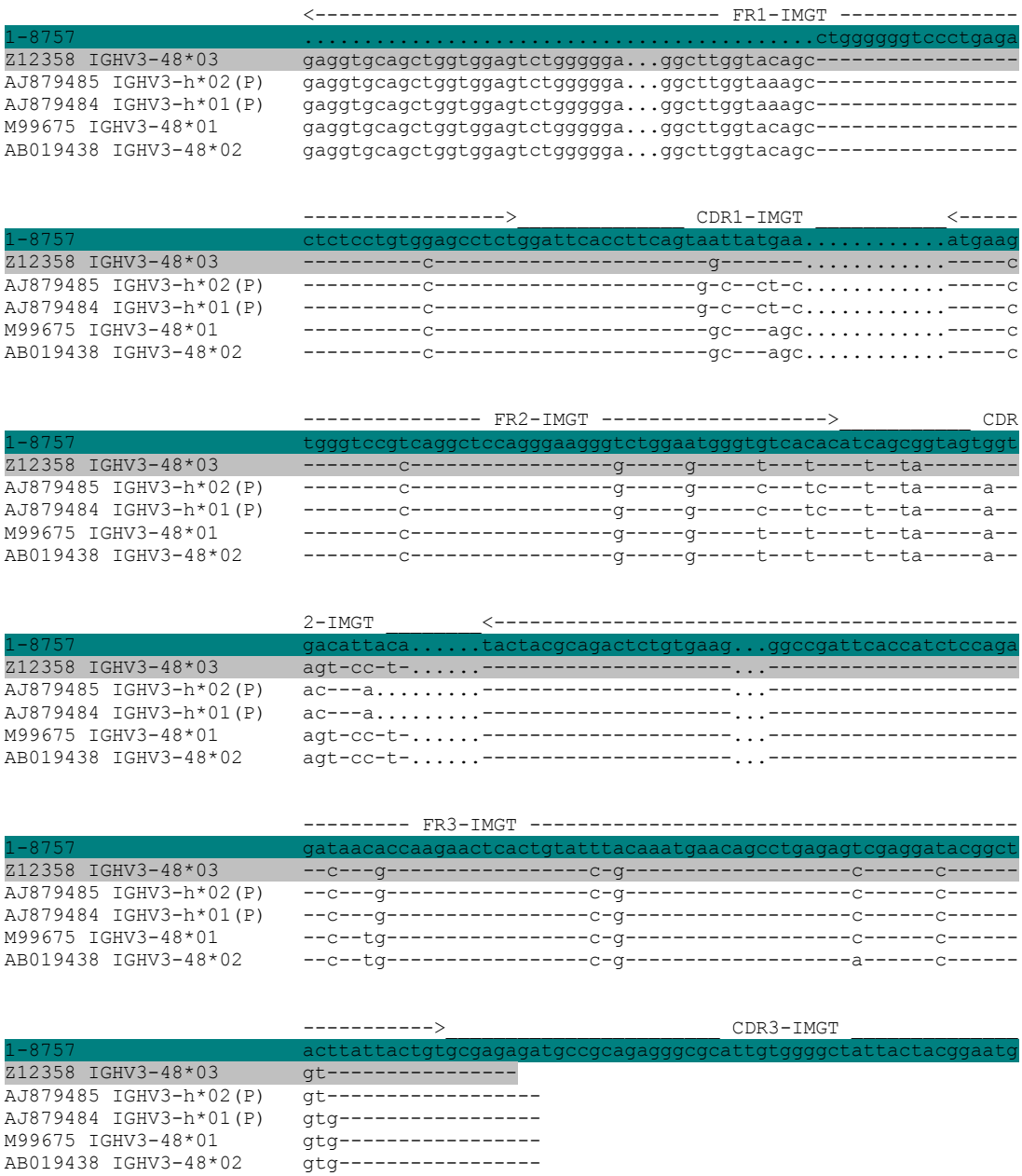

Figure 18: Nucleotide sequence analysis of one *IgV_H* clone (1-8757) of case nr. 9. The corresponding closest germline *IgV_H* gene sequence (VH3-48*03 – highlighted with grey) was determined using the IMGT/V-QUEST program. Sequence homology was 89.91%. The examined clone differed in 25 single nucleotide substitutions compared to the reference sequence. Sequence identity to germline is indicated by a dash ("-") and each mutation is indicated by the appropriate nucleotide.

DOI:10.14753/SE.2012.1744

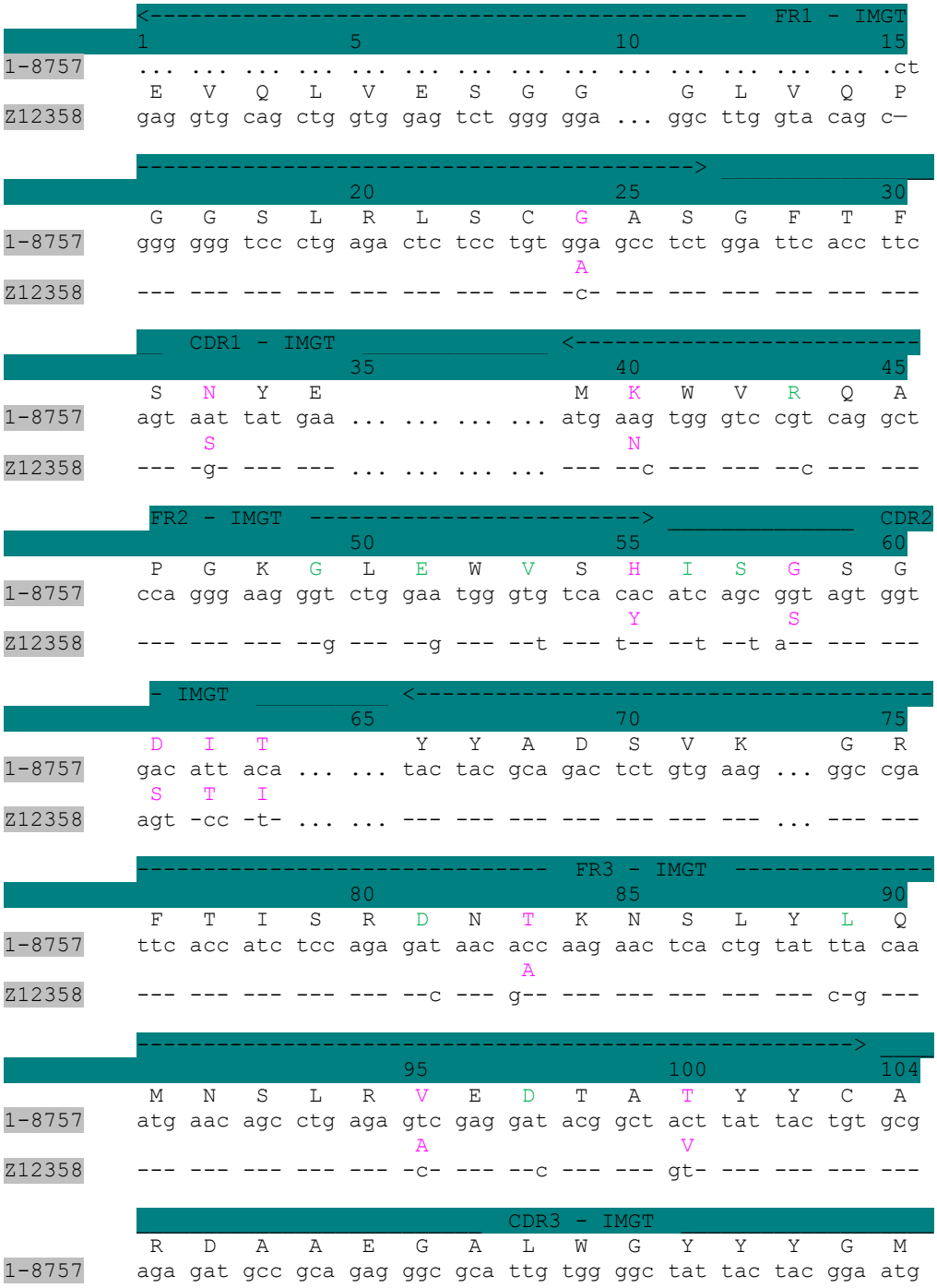

Figure 19: Nucleotide sequence analysis of one *IgV$_H$* clone (1-8757) of case nr. 9. The numbering indicated above the case refers to the codons counted from the beginning of VH. The upper case letters indicate the amino acid translations: with purple color if mutation has led to a replacement amino acid, green color if the mutation was a silent mutation with no amino acid replacement.

DOI:10.14753/SE.2012.1744

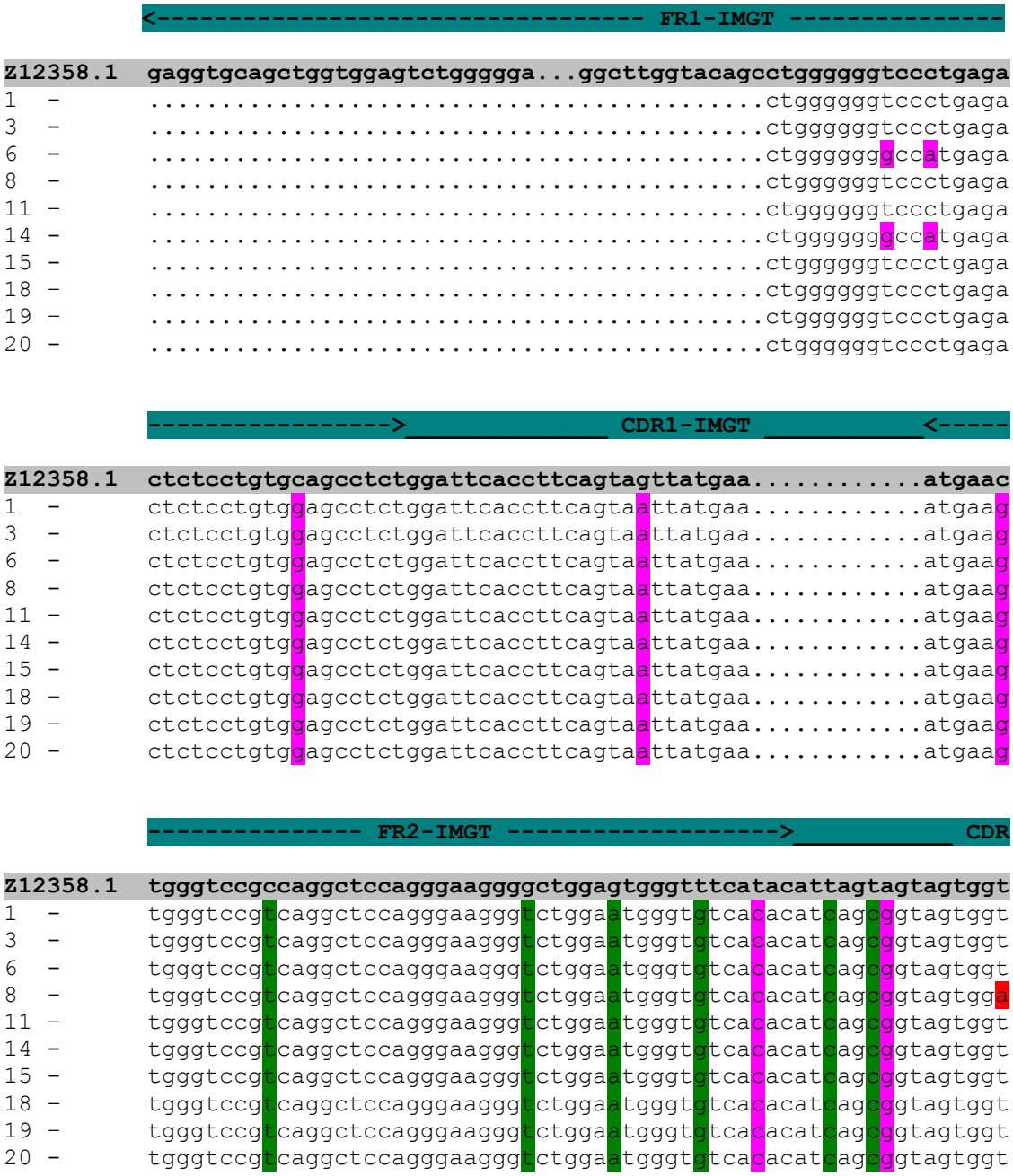

Figure 20 A: Sequences of 10 *IgV_H* gene clones out of 20 in case nr. 9. The represented gene region is highlighted with cyan. The upper sequence highlighted with grey is the closest germline *IgV_H* gene sequence. Numbers in the beginning of each row identify the clones. Nucleotides highlighted with purple color represent mutations that have led to amino acid replacement and nucleotides highlighted with green color represent silent mutations.

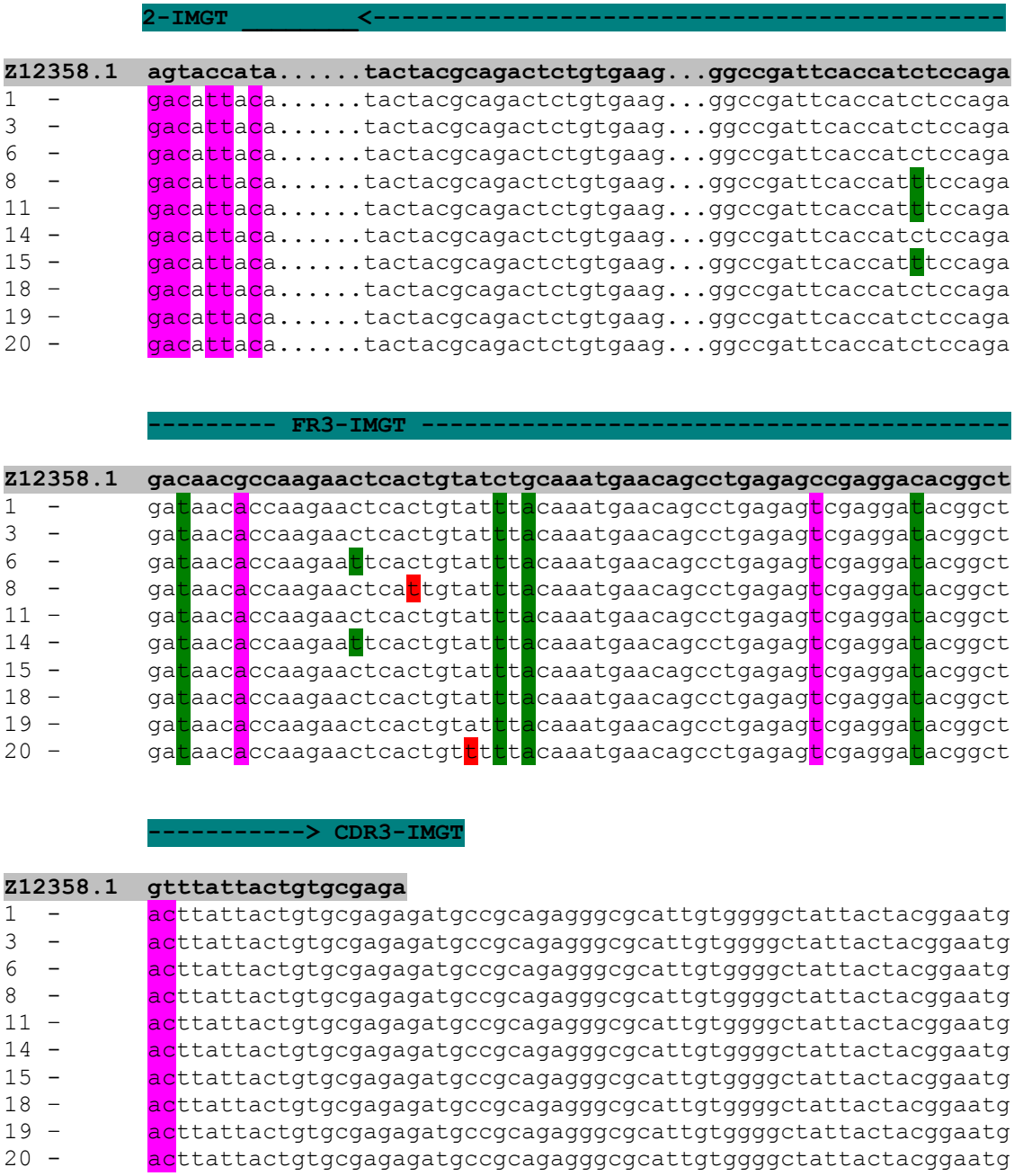

Figure 20 B (continued): Sequences of 10 *IgV*$_H$ gene clones out of 20 in case nr. 9. Mutations found in a single clone only, highlighted with red, were considered Taq enzyme errors and were left out from further analysis. Related intraclonal variations can be observed in clone no. 6 and 14, and in clones 8, 11 and 15, respectively.

DOI:10.14753/SE.2012.1744

Table 13. Analysis of the distribution of somatic mutations of IgV_H genes in follicular lymphoma cases with and without translocation and expression of *BCL2*

Case No.	Closest germline gene	Sequence homology (%)	Mutations /100 bp**	Range of mutation frequency (%)***	Intraclonal divergence No. of clones	CDR1 and CDR2			FR1, FR2 and FR3			P_M
						R	S	P_B-value	R	S	P_B-value	
1	VH3-30*03	90.73-91.12	9.44	8.46-10.48	6	7 (1.16)	2	<0.001*	10-11 (8.01-8.64)	3	0.101-0.127	0.011*-0.17
2	VH4-34*01	86.12-86.53	13.66	13.46-13.87	8	8 (1.06)	1	<0.001*	14 (15.23-15.87)	10-11	0.118-0.142	0.004*-0.028*
3	NA	-	-	-	-	-	-	-	-	-	-	-
4	NA	-	-	-	-	-	-	-	-	-	-	-
5	VH3-23*01	83.06-88.3	13.27	12.5-16.58	4	9-13 (1.51-1.68)	0-3	<0.001*	13-19 (10.39-18.35)	4-11	0.09-0.145	<0.001*-0.074
6	VH3-7*01	92.74-97.58	6.78	2.41-9.67	6	0-2 (0.69)	0-3	<0.001*-0.122	4-10 (3.73-8.11)	2-3	0.081-0.321	<0.001*-0.796
7	VH4-34*01	90.2-90.61	9.73	9.38-9.79	6	7-8 (0.87-0.98)	0	<0.001*	12 (10.1)	4	0.135	0.003*-0.143
8	NA	-	-	-	-	-	-	-	-	-	-	-
9	VH3-48*03	88.7-89.91	11.04	10.48-13.3	7	8 (1.33)	2	<0.001*	7-9 (9.33-11.28)	8-9	0.064-0.101	<0.001*-0.017*
10	VH3-33*01	89.87-90.68	9.76	9.27-10.08	7	4 (0.81-0.95)	2-3	0.003*-0.007*	9-10 (10.65-11.9)	8-9	0.101-0.154	0.003*-0.378
11	VH3-30*03	71.37-71.77	28.41	28.22-28.62	7	13 (2.49)	5	<0.001*	41-42 (31.99-32.61)	11	0.002*-0.003*	0.131-0.338
12	VH4-34*01	89.39-90.2	10.04	9.79-10.61	5	3 (3.1-4.95)	1-2	0.138-0.238	10-11 (13.46-14.95)	8-9	0.046-0.059	0.013*-0.526
13	NA	-	-	-	-	-	-	-	-	-	-	-
14	VH3-48*02	89.11-89.92	10.8	10.08-10.88	3	11 (3.42-3.56)	2	<0.001*	7-9 (15.63-16.88)	5	<0.001*	<0.001*
15	VH3-48*02	86.27-87.12	12.41	12.09-13.33	7	9-10 (3.79-4.09)	2-3	0.003*-0.006*	16-17 (18.6-20.46)	3-5	0.065-0.09	0.003*-0.214
16	VH3-9*01	89.11-90.32	10.56	10.08-11.29	7	10 (3.2-3.59)	1	<0.001*	9-12 (15.47-17.32)	5	0.005*-0.019*	<0.001*-0.023*
17	VH3-9*01	83.87-84.68	15.69	15.32-16.12	4	14 (4.95-5.21)	1	<0.001*	17-18 (23.75-24.82)	6-7	0.011*	<0.001*-0.019*
18	NA	-	-	-	-	-	-	-	-	-	-	-

CDR, complementary determining region; FR, framework region; R, number of observed and (expected) replacement mutations; S, number of detected silent mutations, P_B -value, the probability that this scarcity occurred by chance, according to the Chang and Casali (1994) binomial distribution model; P_M, probability according to the multinomial distribution model of Lossos *et al.* (2000); NA, not amplified. Cases 1-11: BCL2-negative FL, cases 12-18: BCL2-positive FL.

*, statistically significant ($p<0.05$); ** All sequence variants resulted from single nucleotide changes, except for case no. 15. where deletions were also detected, and all of these mutations seemed to represent functional rearrangements since no stop codons or crippling mutations were found; ***The mutation frequency was obtained by dividing the number of mutations by the total number of nucleotides examined.

DOI:10.14753/SE.2012.1744

2. Mutational analysis of *c-MYC, PAX-5* and *RhoH* genes

Eight cases of BCL2-negative FL and seven cases of BCL2-positive FL were subjected to mutational analysis. The position and type of mutations found are reported in Table 14, and the mutation frequencies are shown in Table 15. Figure 21 represents two electropherograms of *c-MYC* exon 2 sequences, in case no. 7 with a C>A mutation and in case no. 2 with no mutation in the same position.

Table 14. Distribution and type of mutations found in *c-MYC*, *PAX-5* and *RhoH* genes in follicular lymphoma cases with and without translocation and expression of *BCL2*. Mutations marked with red represent transitions, mutations marked with blue are transversions.

Case No.	*c-MYC exon 1*	*c-MYC exon 2*	*PAX-5*	*RhoH*
1	3397 (C>T)	-	1087 (C>A)	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	3442 (T>A)	-	-	-
6	3283 (C>T)	-	-	-
7	-	4658 (C>A)	ND	-
8	3542 (C ins)	-	1070 (G>A)	-
9	ND	ND	ND	ND
10	ND	ND	ND	ND
11	ND	ND	ND	ND
12	2406 (A ins)	-	1070 (G>C)	-
13	-	-	1267 (A ins)	-
14	-	-	1265 (G>A)	-
15	-	-	-	-
16	3259 (A>T)	-	-	-
17	-	-	1108 (C>G)	-
18	-	4539 (T>C)	-	-

ND, not determined.

DOI:10.14753/SE.2012.1744

The obtained sequences in case of *c-MYC* exon 1 and exon 2, *PAX-5* and *RhoH* genes were compared to the corresponding germline gene sequences available in the NCBI GenBank database and corresponding positions were used to localize mutations. Previously reported polymorphisms and mutations appearing more than once in separate cases, thus considered as polymorphic variants, were excluded from further analyses. The majority of *c-MYC* mutations were alternatively distributed on 1,5 – ~0,9 kb long fragments in the region downstream to the major P1/P2 (exon 1) or the region downstream to the minor P3 promoter (exon 2). In case of *PAX-5* somatic mutations were identified predominantly around exon 1B, on 1 kb long fragments. No mutations were found in the examined *RhoH* genes.

Totally seven mutations were found in BCL2-positive FL and seven mutations in BCL2-negative FL.

Table 15. Mutational patterns of c-MYC, PAX-5 and RhoH genes in follicular lymphoma cases with and without translocation and expression of BCL2.

Gene	Mutation frequency/100b*	Single bp substitutions	Insertions or deletions	Transitions/ transversions	G+C/A+T	RGYW **
			BCL2-negative FL			
c-MYC exon 1	0,038	3	1	2/1	2/1	0/3
c-MYC exon 2	0,086	1	0	0/1	1/0	0/1
RhoH	0	0	0	0	0	0
PAX-5	0,062	2	0	1/1	2/0	1/2
All genes	-	6	1	3/3	5/1	1/6
			BCL2- positive FL			
c-MYC exon 1	0,038	1	1	0/1	0/1	0/1
c-MYC exon 2	0,086	1	0	1/0	0/1	1/1
RhoH	0	0	0	0	0	0
PAX-5	0,062	3	1	1/2	3/0	2/3
All genes	-	5	2	2/3	3/2	3/5

* Mutation frequencies were calculated on the entire region analyzed and on mutated cases only, taking into account 2 alleles; **RGYW shows number of mutations embedded in this mutational motive (R= A, G; Y= C, T; W= A, T).

DOI:10.14753/SE.2012.1744

The mutation pattern introduced by ASHM corresponds to the specific features of the SHM process seen in physiologic targets. Mutation distribution was similar in the examined two groups: single nucleotide substitutions predominated, with only a few insertions and no deletion. Mutations were distributed in a region spanning up to 2 kb towards the 3' end from the transcription initiation site. There was an equal distribution of transitions (C>T, T>C, G>A) and transversions (T>A, A>T, C>G, G>C, C>A). The ratio of G+C over A+ T substitution was elevated: 5/1 for BCL2-positive FL and 3/2 in case of BCL2-negative FL. A preferential distribution within the RGYW motif (R=A/G, Y=C/T, W=A/T) was observed only in 1 out of 6 single bp substitutions in cases of BCL2-positive FL and in 3 out of 5 mutations in cases of BCL2-negative FL.

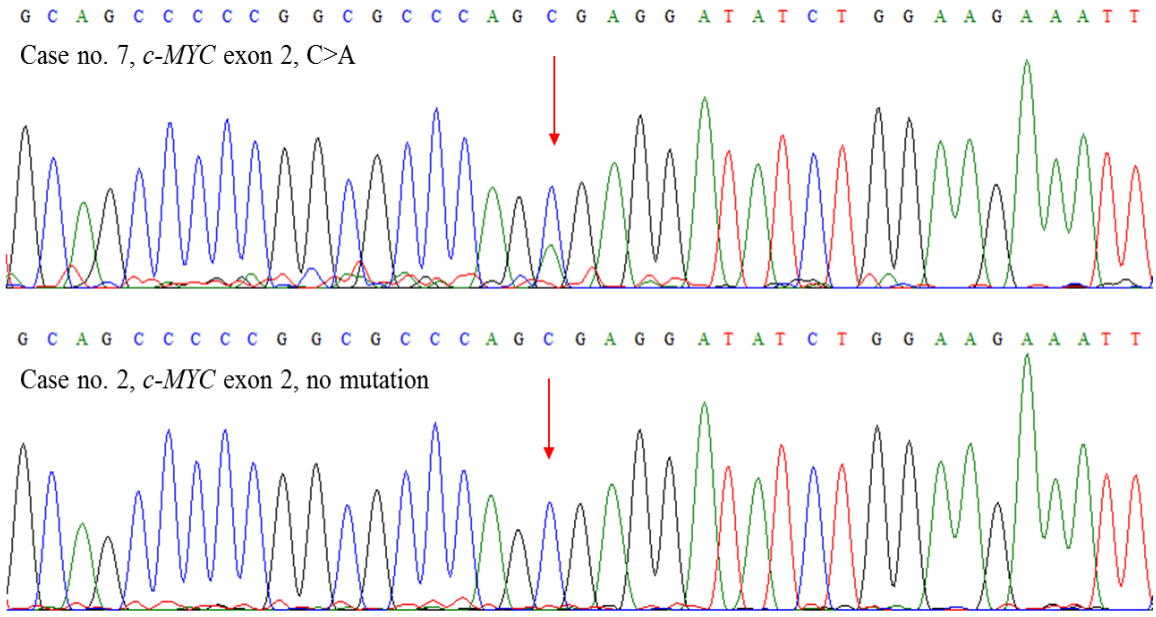

Figure 21: Electropherogram of two *c-MYC* exon 2 sequences. In the first case in addition to the wild type cytosine (blue) an adenine mutation (green) can also be seen, in case no. 2 only the wild type cytosine can be observed.

DOI:10.14753/SE.2012.1744

3. Analysis of AID mRNA expression

To compare the *AID* expression of BCL2-negative and positive FL, we measured the levels of expression of *AID* mRNA in 15 cases of FL (8 BCL2-negative and 7 BCL2-positive), germinal centre B-cells and peripheral blood (PB) mononuclear cells by quantitative real-time PCR assay. The data obtained as relative expression values are shown in Figure 22. These results were calculated as the mean of three independent measurements using the comparative C_T method ($\Delta\Delta C_T$). The levels of *AID* mRNA were defined as a ratio of *AID* to *β-actin* expression.

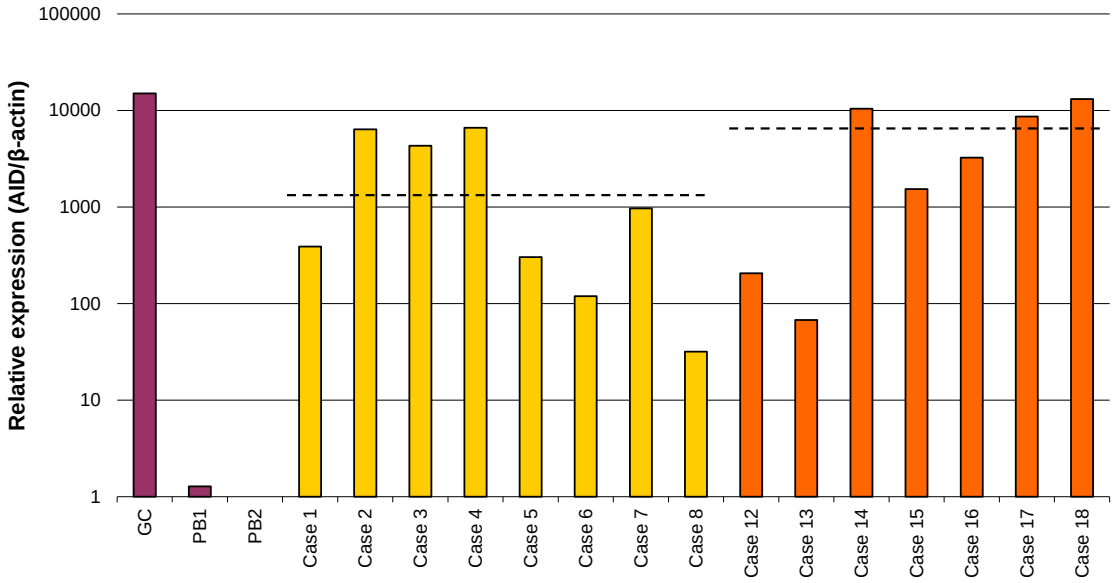

Figure 22: Activation-induced cytidine deaminase (AID) mRNA expression in follicular lymphoma with and without *BCL2* translocation and expression. Quantitative real-time PCR analysis of AID and β-actin was performed on germinal centre B-cells (GC; out of five analysed GC, only one is represented), peripheral blood (PB) mononuclear cells (out of eight analysed PB samples only PB1 and PB2 are shown), BCL2-negative FL (Cases 1-8) and BCL2-positive FL (Cases 12-18). Each column represents the average value of three independent AID/β-actin measurements. The dashed lines represent the average level of the relative expression values of FL without (Cases 1-8) and with (Cases 12-18) the presence of BCL2. The difference is not statistically significant ($p>0.05$).

DOI:10.14753/SE.2012.1744

Mononuclear cells from peripheral blood (PB) of eight healthy volunteers were used as negative controls and as expected a low AID expression was detected. In GC cells microdissected from five reactive lymph nodes, used as positive controls, the measured AID expression level was high. The difference between the average level of relative expression values of follicular lymphoma without and with the presence of BCL2 was statistically not significant ($p>0.05$).

4. Analysis of AID protein expression

AID protein levels were also detected in all 18 FL cases from formalin-fixed paraffin-embedded tissue samples. The results are shown in Table 16.

Table 16. Immunohistochemical data of AID protein expression of patients with and without *BCL2* translocation and expression.

Case No.	Grade	Immunohistochemical analysis	
		BCL2(%)	**AID(%)**
1	3A	-	15
2	3A	-	60
3	2	-	10
4	3A	-	40
5	3A	-	10
6	3A	-	15
7	3A	-	40
8	2	-	10
9	3A	-	15
10	3A	-	50
11	3A	-	10
12	3A	100	50
13	3A	85	80
14	3A	100	35
15	3A	50	40
16	3A	95	30
17	2	60	50
18	2	90	80

Number values indicate the percentage of cells expressing the marker in each sample.

DOI:10.14753/SE.2012.1744

There was no significant difference between the protein expression values of FL with and without the presence of BCL2. The AID protein was predominantly localized in the cytoplasm of germinal centre B-cells as shown in Figure 23.

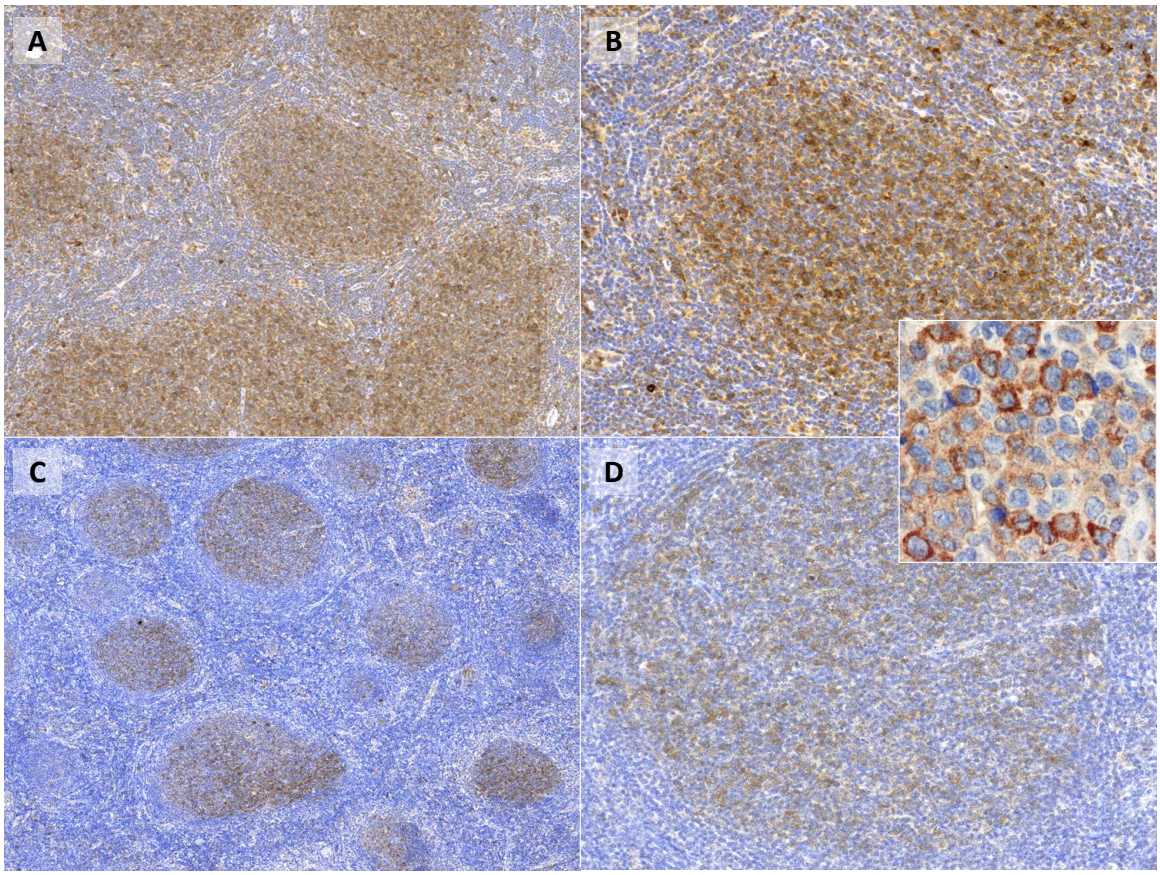

Figure 23: Activation-induced cytidine deaminase (AID) protein expression in follicular lymphoma (FL) with and without *BCL2* translocation and expression. A and B: BCL2-positive FL, C and D: BCL2-negative FL. Insert (400x) showing the predominantly cytoplasmic localization of AID.

No correlation was observed between *AID* mRNA and protein expression levels as shown in Figure 24. Furthermore, no correlation has been found between FL grade, the presence or absence of BCL2 protein and AID expression. Similarly, there was no correlation between AID mRNA and protein expression levels and ASHM mutations in the *c-MYC*, *PAX-5* and *RhoH* genes. However, due to the relative small number of examined cases we were not able to make major conclusions.

DOI:10.14753/SE.2012.1744

Figure 24: AID mRNA and protein expression in follicular lymphoma with and without *BCL2* translocation and expression. No correlation was observed between AID mRNA and protein expression levels.

DOI:10.14753/SE.2012.1744

VI. DISCUSSION

FL are considered to derive from the germinal centre B-cells, based on the immunophenotype of tumour cells, accumulation of somatic hypermutation (SHM) in their immunoglobulin genes and expression of activation-induced (cytidine) deaminase (AID) required for SHM [5, 118].

The t(14;18) translocation and BCL2 overexpression are believed to be strongly associated with the development of FL [44, 45, 48], hence, the absence of these abnormalities in B-cell neoplasms may point to a substantially different transformational pathway and a separate entity.

Several reports suggest that FL without translocation and expression of *BCL2* gene have distinct morphological, genetic and molecular characteristics that distinguish them from the BCL2-positive FL. These BCL2-negative FL are frequently grade 3 lymphomas, however BCL2-negative cases were also observed in lower grade groups [53, 124-127]. Furthermore, grade 3B cases frequently harbour translocation of the *BCL6* gene [128-130], carry trisomy of chromosome 3, and gain of chromosome 18 or 18q [131]. It is currently unclear whether this lymphoma derives from GC or post-GC B-cells. Based on the immunophenotype ($CD10^-$, $MUM1^+$) and frequent *BCL6* gene rearrangement, it has been suggested that these BCL2-negative FL may have a *late-stage* GC or *post*-GC cell origin, and are probably more closely related to the DLBCL [53, 124, 131-133]. However, in a recently published study based on gene expression analysis, Piccaluga *et al.* (2008) [134] suggested that grade 3B FL does after all belong to the group of FL rather than DLBCL. The authors also suggest a possible revision of the histological grading of FL, with their simple distinction into FL (grade 1-3A) and FL/large cell (grade 3B) [134].

To characterize a possible homogenous subtype of FL in which overexpression of *BCL2* gene is not involved, we selected FL cases showing neither the t(14;18) translocation nor the expression of BCL2 protein. Using strict selection criteria, we excluded those cases where tumour cells carried the t(14;18) translocation, but BCL2

expression was lost as a result of somatic mutations of the translocated gene, or cases which lacked the t(14;18) translocation, but BCL2 was overexpressed by different alternative mechanisms [71, 73]. The GC origin of these lymphomas was supported by a follicular growth pattern, the BCL6 and CD10 (except in cases 1 and 5) expression of the tumour cells, and by the presence of CD21 and CD23-positive follicular dendritic reticulum cells within the neoplastic follicles. Morphological analysis of these BCL2-negative FL showed two cases of grade 2 and nine cases of grade 3A FL; there were no cases of grade 3B FL. In our series, only three out of 11 BCL2-negative samples showed *BCL6* gene alterations, in contrast with previous reports, in which a significant fraction of FL without t(14;18) translocation harboured alterations of the *BCL6* gene [126, 130]. However, many *BCL6* breakpoints are localized with several hundreds of kilobases upstream the major breakpoint region, so they could still have been missed in the present series [124]. *BCL6* alterations were also observed in one BCL2-positive FL from the control group.

The SHM process of *Ig* genes is a characteristic feature of FL [135]. IgV_H sequence analysis of FL lacking *BCL2* translocation and expression revealed ongoing somatic mutations generating intraclonal heterogeneity of the tumour clones. The average mutational frequency within the IgV_H region of FL cases without *BCL2* gene rearrangement and expression was 12.76% (range 2.41% to 28.62%) which is similar to the average 11.9% frequency (range 9.79% to 16.12%) found in FL harbouring the t(14;18) translocation. The pattern and distribution of these mutations were highly consistent with antigen selection as calculated by the binomial and the multinomial distribution models [122, 123]. Taken together, these results indicate that cases of FL without *BCL2* gene rearrangement and expression are derived from GC B-cells, and this subtype of FL is not distinct from FL carrying t(14;18) translocation based on the mutational pattern of IgV_H.

According to a widely accepted recent hypothesis a multistep model of tumourgenesis is instrumental in the pathogenesis of FL [48]. The initiating genetic event is the translocation of t(14;18), causing constitutive expression of the anti-apoptotic BCL2, but antigenic stimulation and signalling through the antigen receptor is required

for the neoplastic transformation. Using protein microarray analysis Zha *et al.* (2004) [136] showed that *BCL-X_L* or AKT/BAD pathways may provide an alternative anti-apoptotic signal in FL in the absence of BCL2 protein, and our study may provide evidence that GC microenvironment and antigenic selection are also instrumental in the development of BCL2-negative FL. On this line our research group has recently presented proof on the critical role of reactive tumour microenvironment, i.e. the interaction between the malignant tumour cells and bystander immune cells, in determining FL phenotype and progression including bone marrow involvement [137]. Our data suggest that BCL2-positive and BCL2-negative FL may have different molecular alterations at the starting point of lymphomagenesis, but in both cases the immunoglobulin receptor complex may mediate additional signal(s) required for malignant transformation.

Gene expression analysis of a recent study identified 17 microRNAs that were differentially expressed between t(14;18)-positive FL and t(14;18)-negative FL. The down-regulation of miR-16, miR-26a, miR-101, miR-29c, and miR138 in the t(14;18)-negative FL subset was associated with mRNA expression changes of potential target genes involving cell cycle control, apoptosis, and B-cell differentiation. A major checkpoint kinase, CHEK1, target of miR-16 showed increased expression on the protein level in t(14;18)-negative FL. The CHEK1 up-regulation may contribute to the pro-proliferative phenotype of t(14;18)-negative FL cells and might also confer antiapoptotic properties in FL cells that lack BCL2 expression. Down-regulation of the GC marker TCL1 in BCL2-negative FL, which is expressed in naive and GC B-cells and is down-regulated in post-GC stages of B-cell differentiation was also observed. Thus t(14;18)-negative FL have distinct microRNA profiles that are associated with an increased proliferative capacity and a "late" germinal centre B-cell phenotype [138]. miR-101 was described as tumour suppressor that targets MCL1, which is an antiapoptotic member of the BCL2 family. Moreover, miR-101 and miR-26a were described to inhibit the expression of the polycomb group protein and histone methyl transferase EZH2 in different tumour entities [138]. Recent studies showed that mutations of the *EZH2* are among the most frequent genetic events observed in GC B-cell malignancies after

t(14;18). The mutation *EZH2* Y641 has been shown to behave in a dominant-negative fashion in case of FL, but the exact mechanism by which this mutations contributes to GC lymphomagenesis is yet unknown [139, 140].

SHM occurs during the centroblast stage of B-cell maturation and requires the presence of AID. AID is highly expressed in BCL2-positive FL and DLBCL, in Burkitt lymphoma, mediastinal large B-cell lymphoma and MALT lymphoma [93, 97, 101, 116-118], whereas in healthy B-cell development the expression of AID is highly regulated and restricted to GC B-cells [5]. The constitutive expression of AID may contribute to NHL formation. To analyse whether AID expression is associated with the pathogenesis of BCL2-negative FL, we measured *AID* mRNA expression in eight cases of BCL2-negative FL and compared the results to those of seven BCL2-positive FL. AID mRNA levels were 3 order of magnitude higher in FL than in PB mononuclear cells, they showed various but similar levels in both BCL2-positive and negative FL, but generally did not reach the level detected in normal GC cells. AID expression of FL may reflect the GC origin of the tumour cells as has also been suggested previously by the presence of somatic mutations of IgV_H genes of the tumour cells. This is consistent with previous findings showing coincidence of AID expression with ongoing type of SHM of the *Ig* genes in FL [118]. The fact that we did not find significant differences in *AID* mRNA expression levels between BCL2-positive and negative FL adds further support to the concept that these two variants of FL belong to the same entity.

SHM and AID activity have also been implicated in the alterations of proto-oncogenes such as *PIM-1*, *PAX-5*, *RhoH* and *c-MYC*, which are involved in the pathogenesis of different types of B-cell lymphomas [5, 48]. SHM has been shown to malfunction in about 50% of DLBCL [95], in BCL2-positive FL [96], in mediastinal large B-cell lymphoma [97], in about 20% of AIDS-related NHL [98], in primary central nervous system DLBCL [99], in primary cutaneous marginal zone B-cell lymphoma [100] and in MALT lymphoma [101]. ASHM has also been associated with FL and CLL transformation into higher grade DLBCL [102, 103]. The rates of ASHM we found in FL without *BCL2* rearrangement and expression were lower than previously reported in DLBCL, [95] but similar to those published by others and found by us for BCL2-positive

DOI:10.14753/SE.2012.1744

FL [96, 103], suggesting that both BCL2-positive and negative FL are targeted by a low frequency of ASHM, and BCL2 expression does not influence the incidence of ASHM in FL.

Although, BCL2 protein overexpression is considered to be a critical pathogenic event in the development of FL [44, 46], our findings suggest that FL may develop even without the involvement of the *BCL2* gene. The similarities of morphology, immunophenotype, mutational pattern and AID expression support the hypothesis that besides the initial step of tumourgenesis, BCL2-positive and negative FL (grade 1-3A) represent the same entities with several molecular pathways in common.

DOI:10.14753/SE.2012.1744

VII. CONCLUSIONS

- Our findings suggest that FL may develop even without the involvement of the *BCL2* gene, although, BCL2 protein overexpression is considered to be a critical pathogenic event in the development of FL.

- Our findings demonstrate that follicular lymphomas without *BCL2* gene rearrangement and expression are associated with ongoing type of somatic hypermutation (SHM) of the IgV_H genes, low activity of aberrant somatic hypermutation and elevated mRNA and protein expression of activation-induced cytidine deaminase. These results are in concordance with the results found in the BCL2-positive follicular lymphoma cases.

- These data suggest that BCL2-positive and BCL2-negative FL may have different molecular alterations at the starting point of lymphomagenesis, but in both cases the GC microenvironment and the immunoglobulin receptor complex may provide additional signal(s) required for malignant transformation.

- The present data support the hypothesis that BCL2-positive and BCL2-negative follicular lymphomas (grades 1-3A) after all represent a homogenous group with different initial but several common additional molecular pathways which lead to highly similar morphological, immunophenotypic and also molecular features.

DOI:10.14753/SE.2012.1744

VIII. SUMMARY

Follicular lymphoma is characterized by the t(14;18) translocation resulting in constitutive expression of BCL2 protein; however approximately 10-15% of follicular lymphomas do not express BCL2 protein, and a small fraction of these cases does not exhibit the translocation of the *BCL2* gene either. It is highly debated whether cases of follicular lymphoma without *BCL2* gene rearrangement and expression represent a separate lymphoma entity with distinct biological characteristics, different from the BCL2-positive ones. To further characterize follicular lymphomas without *BCL2* gene rearrangement and expression, we analysed and compared the mutational status of IgV_H genes as well as other genes *(c-MYC*, *PAX-5* and *RhoH)* frequently involved in the specific type of genomic instability called aberrant somatic hypermutation in 11 cases of BCL2-negative and 7 cases of BCL2-positive follicular lymphomas. We also determined the activation-induced cytidine deaminase levels in these cases. The analysed cases were grade 2 and grade 3A follicular lymphomas. Our findings demonstrate that follicular lymphomas without *BCL2* gene rearrangement and expression are associated with ongoing type of somatic hypermutation of the IgV_H genes, low activity of aberrant somatic hypermutation and elevated activation-induced cytidine deaminase expression. These results were in concordance with the results found in the BCL2-positive follicular lymphoma cases. Although, BCL2 protein overexpression is considered to be a critical pathogenic event in the development of follicular lymphoma, our findings suggest that follicular lymphomas with the same morphology, immunophenotype, mutational pattern and activation-induced cytidine deaminase expression may develop without the involvement of *BCL2* gene. The present data support the hypothesis that BCL2-positive and BCL2-negative follicular lymphomas (grades 1-3A) represent a homogenous group with different initial but several common additional molecular pathways.

DOI:10.14753/SE.2012.1744

IX. ÖSSZEFOGLALÁS

A folliculáris lymphomák (FL) klasszikus jellemzője a t(14;18)-as kromoszómatranszlokáció, amelyben a BCL2 fehérje fokozott expressziója figyelhető meg. Irodalmi adatok alapján azonban a FL-ák 10-15%-ban nem mutatható ki BCL2 overexpresszió, és az esetek 2-5%-ban a *BCL2* gént érintő transzlokáció sem azonosítható. Ezen esetek leggyakrabban a grade 3-as FL-ák csoportjába tartoznak, amelyek eltérő molekuláris és genetikai jellemzőik alapján elkülönülnek a többi FL-tól. Ez okból egyes szerzők a WHO klasszifikáció önálló entitásaként való besorolását javasolják. Célkitűzésünk, hogy további adatokat szolgáltassunk ezen entitások patogenezisében szerepet játszó genetikai elváltozásokról. Ennek érdekében megvizsgáltuk és összehasonlítottuk az aberráns szomatikus hipermutáció (ASHM) által érintett *PAX-5, RhoH/TTF* és *c-MYC* gének mutációs státusát, az Ig nehézlánc (IgV_H) gének szomatikus hipermutációs mintázatát, valamint az aktiváció-indukált citidin deamináz (AID) mRNS és fehérje expressziós szinteket 11 BCL2-negatív és 7 BCL2-pozitív folliculáris lymphomában. A vizsgált esetek nagyrészében az ASHM által érintett onkogének mutációt hordoztak, magas AID expressziót mutattak, ugyanakkor a tumorsejtek IgV_H génjeit ongoing jellegű SHM jellemezte. Következtetésként elmondhatjuk, hogy a BCL2-negatív folliculáris lymphomákban úgy a SHM mint az ASHM aktivitása megfigyelhető. Ugyanakkor, a t(14;18)-as transzlokáció és BCL2 overexpresszió sem szükséges, sem elégséges feltételnek nem tekinthető a FL patogenezisének szempontjából, hiszen hasonló morfológiájú, immunfenotípusú, azonos mutációs mintázattal és AID aktivitással rendelkező folliculáris lymphomák alakulhatnak ki a *BCL2* gén érintettsége nélkül is. A BCL2-negatív és BCL2-pozitív folliculáris lymphoma prekurzor-sejteknek egyaránt szükségük van a nyirokcsomói mikrokörnyezet ingereire, valamint az Ig-receptorkomplex mediálta antigén szelekcióra a malignus transzformációhoz. Eredményeink alapján a BCL2-negatív FL és BCL2-pozitív FL (grade 1-3A) egyazon entitás két altípusa.

DOI:10.14753/SE.2012.1744

X. REFERENCES

1. Jaffe, E.S., N.L. Harris, H. Stein, E. Campo, S.A. Pileri, and S.H. Swerdlow, *Introduction and overview of the classification of the lymphoid neoplasms*, in *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, S.H. Swerdlow, E. Campo, N.L. Harris, E.S. Jaffe, S.A. Pileri, H. Stein, J. Thiele, and J.W. Vardiman, Editors. 2008, IARC: Lyon. p. 158-166.
2. Campo, E., S.H. Swerdlow, N.L. Harris, S. Pileri, H. Stein, and E.S. Jaffe, *The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications.* Blood, 2011. **117**(19): p. 5019-32.
3. Nogai, H., B. Dorken, and G. Lenz, *Pathogenesis of non-Hodgkin's lymphoma.* J Clin Oncol, 2011. **29**(14): p. 1803-11.
4. Borowitz, M.J. and J.K.C. Chan, *B lymphoblastic leukaemia/lymphoma, not otherwise specified*, in *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, S.H. Swerdlow, E. Campo, N.L. Harris, E.S. Jaffe, S.A. Pileri, H. Stein, J. Thiele, and J.W. Vardiman, Editors. 2008, IARC: Lyon. p. 168-170.
5. Natkunam, Y., *The biology of the germinal center.* Hematology Am Soc Hematol Educ Program, 2007. **2007**: p. 210-5.
6. Klein, U., G. Klein, B. Ehlin-Henriksson, K. Rajewsky, and R. Kuppers, *Burkitt's lymphoma is a malignancy of mature B cells expressing somatically mutated V region genes.* Mol Med, 1995. **1**(5): p. 495-505.
7. Kuppers, R., M.L. Hansmann, and K. Rajewsky, *Clonality and germinal centre B-cell derivation of Hodgkin/Reed-Sternberg cells in Hodgkin's disease.* Ann Oncol, 1998. **9 Suppl 5**: p. S17-20.
8. Kuppers, R., K. Rajewsky, and M.L. Hansmann, *Diffuse large cell lymphomas are derived from mature B cells carrying V region genes with a high load of somatic mutation and evidence of selection for antibody expression.* Eur J Immunol, 1997. **27**(6): p. 1398-405.

9. Staudt, L.M., *A closer look at follicular lymphoma.* N Engl J Med, 2007. **356**(7): p. 741-2.
10. Ghia, P. and F. Caligaris-Cappio, *The origin of B-cell chronic lymphocytic leukemia.* Semin Oncol, 2006. **33**(2): p. 150-6.
11. Ho, P.J., L.J. Campbell, J. Gibson, R. Brown, and D. Joshua, *The biology and cytogenetics of multiple myeloma.* Rev Clin Exp Hematol, 2002. **6**(3): p. 276-300.
12. Kuppers, R. and K. Rajewsky, *The origin of Hodgkin and Reed/Sternberg cells in Hodgkin's disease.* Annu Rev Immunol, 1998. **16**: p. 471-93.
13. Leich, E., G. Ott, and A. Rosenwald, *Pathology, pathogenesis and molecular genetics of follicular NHL.* Best Pract Res Clin Haematol, 2011. **24**(2): p. 95-109.
14. Anderson, J.R., J.O. Armitage, and D.D. Weisenburger, *Epidemiology of the non-Hodgkin's lymphomas: distributions of the major subtypes differ by geographic locations. Non-Hodgkin's Lymphoma Classification Project.* Ann Oncol, 1998. **9**(7): p. 717-20.
15. *A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project.* Blood, 1997. **89**(11): p. 3909-18.
16. Finn, L.S., D.S. Viswanatha, J.B. Belasco, H. Snyder, D. Huebner, L. Sorbara, M. Raffeld, E.S. Jaffe, and K.E. Salhany, *Primary follicular lymphoma of the testis in childhood.* Cancer, 1999. **85**(7): p. 1626-35.
17. Lorsbach, R.B., D. Shay-Seymore, J. Moore, P.M. Banks, R.P. Hasserjian, J.T. Sandlund, and F.G. Behm, *Clinicopathologic analysis of follicular lymphoma occurring in children.* Blood, 2002. **99**(6): p. 1959-64.
18. Pinto, A., R.E. Hutchison, L.H. Grant, C.L. Trevenen, and C.W. Berard, *Follicular lymphomas in pediatric patients.* Mod Pathol, 1990. **3**(3): p. 308-13.
19. Swerdlow, S.H., *Pediatric follicular lymphomas, marginal zone lymphomas, and marginal zone hyperplasia.* Am J Clin Pathol, 2004. **122 Suppl**: p. S98-109.
20. Harris, N.L., S.H. Swerdlow, E.S. Jaffe, G. Ott, B.N. Nathwani, D. de Jong, T. Yoshino, and D. Spagnolo, *Follicular lymphoma*, in *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.*, S.H. Swerdlow, E. Campo,

N.L. Harris, E.S. Jaffe, S.A. Pileri, H. Stein, J. Thiele, and J.W. Vardiman, Editors. 2008, IARC Press: Lyon. p. 220-226.

21. Aster, J.C., *Diseases of White Blood Cells, Lymph Nodes, Spleen, and Thymus*, in *Robbins and Cotran Pathologic Basis of Disease*, V. Kumar, A.K. Abbas, and N. Fausto, Editors. 2005, Elsevier Saunders. p. 674-676.
22. Cullen, M.H., T.A. Lister, R.I. Brearley, W.S. Shand, and A.G. Stansfeld, *Histological transformation of non-Hodgkin's lymphoma: a prospective study.* Cancer, 1979. **44**(2): p. 645-51.
23. Garvin, A.J., R.M. Simon, C.K. Osborne, J. Merrill, R.C. Young, and C.W. Berard, *An autopsy study of histologic progression in non-Hodgkin's lymphomas. 192 cases from the National Cancer Institute.* Cancer, 1983. **52**(3): p. 393-8.
24. Hitz, F., N. Ketterer, A. Lohri, U. Mey, S. Pederiva, C. Renner, C. Taverna, A. Hartmann, K. Yeow, S. Bodis, and E. Zucca, *Diagnosis and treatment of follicular lymphoma.* Swiss Med Wkly, 2011. **141**: p. w13247.
25. Martin, A.R., D.D. Weisenburger, W.C. Chan, E.I. Ruby, J.R. Anderson, J.M. Vose, P.J. Bierman, M.A. Bast, D.T. Daley, and J.O. Armitage, *Prognostic value of cellular proliferation and histologic grade in follicular lymphoma.* Blood, 1995. **85**(12): p. 3671-8.
26. Kantarjian, H.M., P. McLaughlin, L.M. Fuller, D.O. Dixon, B.M. Osborne, and F.F. Cabanillas, *Follicular large cell lymphoma: analysis and prognostic factors in 62 patients.* J Clin Oncol, 1984. **2**(7): p. 811-9.
27. Jones, S.E., Z. Fuks, M. Bull, M.E. Kadin, R.F. Dorfman, H.S. Kaplan, S.A. Rosenberg, and H. Kim, *Non-Hodgkin's lymphomas. IV. Clinicopathologic correlation in 405 cases.* Cancer, 1973. **31**(4): p. 806-23.
28. Gallagher, C.J., W.M. Gregory, A.E. Jones, A.G. Stansfeld, M.A. Richards, H.S. Dhaliwal, J.S. Malpas, and T.A. Lister, *Follicular lymphoma: prognostic factors for response and survival.* J Clin Oncol, 1986. **4**(10): p. 1470-80.
29. Klein, U., T. Goossens, M. Fischer, H. Kanzler, A. Braeuninger, K. Rajewsky, and R. Kuppers, *Somatic hypermutation in normal and transformed human B cells.* Immunol Rev, 1998. **162**: p. 261-80.

30. Bahler, D.W. and R. Levy, *Clonal evolution of a follicular lymphoma: evidence for antigen selection.* Proc Natl Acad Sci U S A, 1992. **89**(15): p. 6770-4.
31. Berek, C., A. Berger, and M. Apel, *Maturation of the immune response in germinal centers.* Cell, 1991. **67**(6): p. 1121-9.
32. Liu, Y.J., D.E. Joshua, G.T. Williams, C.A. Smith, J. Gordon, and I.C. MacLennan, *Mechanism of antigen-driven selection in germinal centres.* Nature, 1989. **342**(6252): p. 929-31.
33. Both, G.W., L. Taylor, J.W. Pollard, and E.J. Steele, *Distribution of mutations around rearranged heavy-chain antibody variable-region genes.* Mol Cell Biol, 1990. **10**(10): p. 5187-96.
34. Stamatopoulos, K., C. Kosmas, C. Belessi, N. Stavroyianni, P. Kyriazopoulos, and T. Papadaki, *Molecular insights into the immunopathogenesis of follicular lymphoma.* Immunol Today, 2000. **21**(6): p. 298-305.
35. Levy, S., E. Mendel, S. Kon, Z. Avnur, and R. Levy, *Mutational hot spots in Ig V region genes of human follicular lymphomas.* J Exp Med, 1988. **168**(2): p. 475-89.
36. Oeschger, S., A. Brauninger, R. Kuppers, and M.L. Hansmann, *Tumor cell dissemination in follicular lymphoma.* Blood, 2002. **99**(6): p. 2192-8.
37. Levy, R., S. Levy, M.L. Cleary, W. Carroll, S. Kon, J. Bird, and J. Sklar, *Somatic mutation in human B-cell tumors.* Immunol Rev, 1987. **96**: p. 43-58.
38. Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo, *Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme.* Cell, 2000. **102**(5): p. 553-63.
39. Muto, T., M. Muramatsu, M. Taniwaki, K. Kinoshita, and T. Honjo, *Isolation, tissue distribution, and chromosomal localization of the human activation-induced cytidine deaminase (AID) gene.* Genomics, 2000. **68**(1): p. 85-8.
40. Martin, A. and M.D. Scharff, *Somatic hypermutation of the AID transgene in B and non-B cells.* Proc Natl Acad Sci U S A, 2002. **99**(19): p. 12304-8.

41. Martin, A. and M.D. Scharff, *AID and mismatch repair in antibody diversification.* Nat Rev Immunol, 2002. **2**(8): p. 605-14.
42. Oviatt, D.L., J.B. Cousar, R.D. Collins, J.M. Flexner, and R.S. Stein, *Malignant lymphomas of follicular center cell origin in humans. V. Incidence, clinical features, and prognostic implications of transformation of small cleaved cell nodular lymphoma.* Cancer, 1984. **53**(5): p. 1109-14.
43. Szereday, Z., B. Csernus, M. Nagy, T. Laszlo, R.A. Warnke, and A. Matolcsy, *Somatic mutation of the 5' noncoding region of the BCL-6 gene is associated with intraclonal diversity and clonal selection in histological transformation of follicular lymphoma.* Am J Pathol, 2000. **156**(3): p. 1017-24.
44. McDonnell, T.J., N. Deane, F.M. Platt, G. Nunez, U. Jaeger, J.P. McKearn, and S.J. Korsmeyer, *bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation.* Cell, 1989. **57**(1): p. 79-88.
45. Tsujimoto, Y., J. Cossman, E. Jaffe, and C.M. Croce, *Involvement of the bcl-2 gene in human follicular lymphoma.* Science, 1985. **228**(4706): p. 1440-3.
46. Vaux, D.L., S. Cory, and J.M. Adams, *Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells.* Nature, 1988. **335**(6189): p. 440-2.
47. de Jong, D., *Molecular pathogenesis of follicular lymphoma: a cross talk of genetic and immunologic factors.* J Clin Oncol, 2005. **23**(26): p. 6358-63.
48. Bende, R.J., L.A. Smit, and C.J. van Noesel, *Molecular pathways in follicular lymphoma.* Leukemia, 2007. **21**(1): p. 18-29.
49. Summers, K.E., L.K. Goff, A.G. Wilson, R.K. Gupta, T.A. Lister, and J. Fitzgibbon, *Frequency of the Bcl-2/IgH rearrangement in normal individuals: implications for the monitoring of disease in patients with follicular lymphoma.* J Clin Oncol, 2001. **19**(2): p. 420-4.
50. Au, W.Y., D.E. Horsman, R.D. Gascoyne, D.S. Viswanatha, R.J. Klasa, and J.M. Connors, *The spectrum of lymphoma with 8q24 aberrations: a clinical, pathological and cytogenetic study of 87 consecutive cases.* Leuk Lymphoma, 2004. **45**(3): p. 519-28.

51. Voorhees, P.M., K.A. Carder, S.V. Smith, L.H. Ayscue, K.W. Rao, and C.H. Dunphy, *Follicular lymphoma with a burkitt translocation--predictor of an aggressive clinical course: a case report and review of the literature.* Arch Pathol Lab Med, 2004. **128**(2): p. 210-3.

52. Katzenberger, T., G. Ott, T. Klein, J. Kalla, H.K. Muller-Hermelink, and M.M. Ott, *Cytogenetic alterations affecting BCL6 are predominantly found in follicular lymphomas grade 3B with a diffuse large B-cell component.* Am J Pathol, 2004. **165**(2): p. 481-90.

53. Ott, G., T. Katzenberger, A. Lohr, S. Kindelberger, T. Rudiger, M. Wilhelm, J. Kalla, A. Rosenwald, J.G. Muller, M.M. Ott, and H.K. Muller-Hermelink, *Cytomorphologic, immunohistochemical, and cytogenetic profiles of follicular lymphoma: 2 types of follicular lymphoma grade 3.* Blood, 2002. **99**(10): p. 3806-12.

54. Hoglund, M., L. Sehn, J.M. Connors, R.D. Gascoyne, R. Siebert, T. Sall, F. Mitelman, and D.E. Horsman, *Identification of cytogenetic subgroups and karyotypic pathways of clonal evolution in follicular lymphomas.* Genes Chromosomes Cancer, 2004. **39**(3): p. 195-204.

55. Offit, K., N.Z. Parsa, G. Gaidano, D.A. Filippa, D. Louie, D. Pan, S.C. Jhanwar, R. Dalla-Favera, and R.S. Chaganti, *6q deletions define distinct clinico-pathologic subsets of non-Hodgkin's lymphoma.* Blood, 1993. **82**(7): p. 2157-62.

56. Tilly, H., A. Rossi, A. Stamatoullas, B. Lenormand, C. Bigorgne, A. Kunlin, M. Monconduit, and C. Bastard, *Prognostic value of chromosomal abnormalities in follicular lymphoma.* Blood, 1994. **84**(4): p. 1043-9.

57. Horsman, D.E., J.M. Connors, T. Pantzar, and R.D. Gascoyne, *Analysis of secondary chromosomal alterations in 165 cases of follicular lymphoma with t(14;18).* Genes Chromosomes Cancer, 2001. **30**(4): p. 375-82.

58. Viardot, A., T.F. Barth, P. Moller, H. Dohner, and M. Bentz, *Cytogenetic evolution of follicular lymphoma.* Semin Cancer Biol, 2003. **13**(3): p. 183-90.

59. Mohamed, A.N., M. Palutke, L. Eisenberg, and A. Al-Katib, *Chromosomal analyses of 52 cases of follicular lymphoma with t(14;18), including blastic/blastoid variant.* Cancer Genet Cytogenet, 2001. **126**(1): p. 45-51.

60. Graninger, W.B., M. Seto, B. Boutain, P. Goldman, and S.J. Korsmeyer, *Expression of Bcl-2 and Bcl-2-Ig fusion transcripts in normal and neoplastic cells.* J Clin Invest, 1987. **80**(5): p. 1512-5.

61. Ott, G. and A. Rosenwald, *Molecular pathogenesis of follicular lymphoma.* Haematologica, 2008. **93**(12): p. 1773-6.

62. Tsujimoto, Y., L.R. Finger, J. Yunis, P.C. Nowell, and C.M. Croce, *Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation.* Science, 1984. **226**(4678): p. 1097-9.

63. Tsujimoto, Y., J. Gorham, J. Cossman, E. Jaffe, and C.M. Croce, *The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining.* Science, 1985. **229**(4720): p. 1390-3.

64. Yunis, J.J., M.M. Oken, M.E. Kaplan, K.M. Ensrud, R.R. Howe, and A. Theologides, *Distinctive chromosomal abnormalities in histologic subtypes of non-Hodgkin's lymphoma.* N Engl J Med, 1982. **307**(20): p. 1231-6.

65. Bordeleau, L. and N.L. Berinstein, *Molecular diagnostics in follicular non-Hodgkin's lymphoma: a review.* Semin Oncol, 2000. **27**(6 Suppl 12): p. 42-52.

66. Liu, J., R.M. Johnson, and S.T. Traweek, *Rearrangement of the BCL-2 gene in follicular lymphoma. Detection by PCR in both fresh and fixed tissue samples.* Diagn Mol Pathol, 1993. **2**(4): p. 241-7.

67. Ngan, B.Y., J. Nourse, and M.L. Cleary, *Detection of chromosomal translocation t(14;18) within the minor cluster region of bcl-2 by polymerase chain reaction and direct genomic sequencing of the enzymatically amplified DNA in follicular lymphomas.* Blood, 1989. **73**(7): p. 1759-62.

68. Cleary, M.L., N. Galili, and J. Sklar, *Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas.* J Exp Med, 1986. **164**(1): p. 315-20.

69. Hillion, J., C. Mecucci, A. Aventin, D. Leroux, I. Wlodarska, H. Van Den Berghe, and C.J. Larsen, *A variant translocation t(2;18) in follicular lymphoma involves the 5' end of bcl-2 and Ig kappa light chain gene.* Oncogene, 1991. **6**(1): p. 169-72.

70. Buchonnet, G., F. Jardin, N. Jean, P. Bertrand, F. Parmentier, S. Tison, S. Lepretre, N. Contentin, P. Lenain, A. Stamatoullas-Bastard, H. Tilly, and C. Bastard, *Distribution of BCL2 breakpoints in follicular lymphoma and correlation with clinical features: specific subtypes or same disease?* Leukemia, 2002. **16**(9): p. 1852-6.

71. Aster, J.C. and J.A. Longtine, *Detection of BCL2 rearrangements in follicular lymphoma.* Am J Pathol, 2002. **160**(3): p. 759-63.

72. Yang, E. and S.J. Korsmeyer, *Molecular thanatopsis: a discourse on the BCL2 family and cell death.* Blood, 1996. **88**(2): p. 386-401.

73. Schraders, M., D. de Jong, P. Kluin, P. Groenen, and H. van Krieken, *Lack of Bcl-2 expression in follicular lymphoma may be caused by mutations in the BCL2 gene or by absence of the t(14;18) translocation.* J Pathol, 2005. **205**(3): p. 329-35.

74. Michallet, A.S., B. Coiffier, and G. Salles, *Maintenance therapy in follicular lymphoma.* Curr Opin Oncol, 2011. **23**(5): p. 449-54.

75. Horning, S.J. and S.A. Rosenberg, *The natural history of initially untreated low-grade non-Hodgkin's lymphomas.* N Engl J Med, 1984. **311**(23): p. 1471-5.

76. Ardeshna, K.M., P. Smith, A. Norton, B.W. Hancock, P.J. Hoskin, K.A. MacLennan, R.E. Marcus, A. Jelliffe, G. Vaughan, Hudson, and D.C. Linch, *Long-term effect of a watch and wait policy versus immediate systemic treatment for asymptomatic advanced-stage non-Hodgkin lymphoma: a randomised controlled trial.* Lancet, 2003. **362**(9383): p. 516-22.

77. www.nccn.org.

78. http://prima.gela.org/.

79. Olin, R.L., P.A. Kanetsky, T.R. Ten Have, S.D. Nasta, S.J. Schuster, and C. Andreadis, *Determinants of the optimal first-line therapy for follicular lymphoma: a decision analysis.* Am J Hematol, 2010. **85**(4): p. 255-60.

80. Marcus, R., K. Imrie, P. Solal-Celigny, J.V. Catalano, A. Dmoszynska, J.C. Raposo, F.C. Offner, J. Gomez-Codina, A. Belch, D. Cunningham, E. Wassner-Fritsch, and G. Stein, *Phase III study of R-CVP compared with cyclophosphamide, vincristine, and prednisone alone in patients with previously untreated advanced follicular lymphoma.* J Clin Oncol, 2008. **26**(28): p. 4579-86.

81. Friedberg, J.W., M.D. Taylor, J.R. Cerhan, C.R. Flowers, H. Dillon, C.M. Farber, E.S. Rogers, J.D. Hainsworth, E.K. Wong, J.M. Vose, A.D. Zelenetz, and B.K. Link, *Follicular lymphoma in the United States: first report of the national LymphoCare study.* J Clin Oncol, 2009. **27**(8): p. 1202-8.

82. Rummel, M.J., N. Niederle, G. Maschmeyer, A. Banat, U.v. Gruenhagen, C. Losem, G. Heil, M. Welslau, C. Balser, U. Kaiser, H. Ballo, E. Weidmann, H.A. Duerk, D. Kofahl-Krause, F. Roller, J. Barth, D. Hoelzer, A. Hinke, and W. Brugger, *Bendamustine Plus Rituximab Is Superior in Respect of Progression Free Survival and CR Rate When Compared to CHOP Plus Rituximab as First-Line Treatment of Patients with Advanced Follicular, Indolent, and Mantle Cell Lymphomas: Final Results of a Randomized Phase III Study of the StiL (Study Group Indolent Lymphomas, Germany).* Blood, 2009. **114**: p. abstract 405.

83. Deconinck, E., C. Foussard, N. Milpied, P. Bertrand, P. Michenet, P. Cornillet-LeFebvre, M. Escoffre-Barbe, H. Maisonneuve, V. Delwail, R. Gressin, E. Legouffe, J.P. Vilque, B. Desablens, J. Jaubert, J.F. Ramee, A. Jenabian, A. Thyss, A. Le Pourhiet-Le Mevel, P. Travade, R. Delepine, and P. Colombat, *High-dose therapy followed by autologous purged stem-cell transplantation and doxorubicin-based chemotherapy in patients with advanced follicular lymphoma: a randomized multicenter study by GOELAMS.* Blood, 2005. **105**(10): p. 3817-23.

84. Gribben, J.G., *How I treat indolent lymphoma.* Blood, 2007. **109**(11): p. 4617-26.

85. Freedman, A., *Follicular lymphoma: 2011 update on diagnosis and management.* Am J Hematol, 2011. **86**(9): p. 768-75.

86. Federico, M., M. Bellei, L. Marcheselli, S. Luminari, A. Lopez-Guillermo, U. Vitolo, B. Pro, S. Pileri, A. Pulsoni, P. Soubeyran, S. Cortelazzo, G. Martinelli, M. Martelli, L. Rigacci, L. Arcaini, F. Di Raimondo, F. Merli, E. Sabattini, P. McLaughlin, and P. Solal-Celigny, *Follicular lymphoma international prognostic index 2: a new prognostic index for follicular lymphoma developed by the international follicular lymphoma prognostic factor project.* J Clin Oncol, 2009. **27**(27): p. 4555-62.

87. Lengauer, C., K.W. Kinzler, and B. Vogelstein, *Genetic instabilities in human cancers.* Nature, 1998. **396**(6712): p. 643-9.

88. Anderson, G.R., *Genomic instability in cancer.* Current Science, 2001. **81**(5): p. 501-507.

89. Scarpa, A., P.S. Moore, G. Rigaud, G. Inghirami, M. Montresor, M. Menegazzi, G. Todeschini, and F. Menestrina, *Molecular features of primary mediastinal B-cell lymphoma: involvement of p16INK4A, p53 and c-myc.* Br J Haematol, 1999. **107**(1): p. 106-13.

90. Fulop, Z., B. Csernus, B. Timar, A. Szepesi, and A. Matolcsy, *Microsatellite instability and hMLH1 promoter hypermethylation in Richter's transformation of chronic lymphocytic leukemia.* Leukemia, 2003. **17**(2): p. 411-5.

91. Nagy, M., M. Balazs, Z. Adam, Z. Petko, B. Timar, Z. Szereday, T. Laszlo, R.A. Warnke, and A. Matolcsy, *Genetic instability is associated with histological transformation of follicle center lymphoma.* Leukemia, 2000. **14**(12): p. 2142-8.

92. Raptis, S. and B. Bapat, *Genetic instability in human tumors.* EXS, 2006(96): p. 303-20.

93. Pasqualucci, L., R. Guglielmino, J. Houldsworth, J. Mohr, S. Aoufouchi, R. Polakiewicz, R.S. Chaganti, and R. Dalla-Favera, *Expression of the AID protein in normal and neoplastic B cells.* Blood, 2004. **104**(10): p. 3318-25.

94. Pasqualucci, L., A. Migliazza, N. Fracchiolla, C. William, A. Neri, L. Baldini, R.S. Chaganti, U. Klein, R. Kuppers, K. Rajewsky, and R. Dalla-Favera, *BCL-6 mutations in normal germinal center B cells: evidence of somatic hypermutation acting outside Ig loci.* Proc Natl Acad Sci U S A, 1998. **95**(20): p. 11816-21.

95. Pasqualucci, L., P. Neumeister, T. Goossens, G. Nanjangud, R.S. Chaganti, R. Kuppers, and R. Dalla-Favera, *Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas.* Nature, 2001. **412**(6844): p. 341-6.

96. Halldorsdottir, A.M., M. Fruhwirth, A. Deutsch, A. Aigelsreiter, C. Beham-Schmid, B.A. Agnarsson, P. Neumeister, and W. Richard Burack, *Quantifying the role of aberrant somatic hypermutation in transformation of follicular lymphoma.* Leuk Res, 2008.

97. Bodor, C., A. Bognar, L. Reiniger, A. Szepesi, E. Toth, L. Kopper, and A. Matolcsy, *Aberrant somatic hypermutation and expression of activation-induced cytidine deaminase mRNA in mediastinal large B-cell lymphoma.* Br J Haematol, 2005. **129**(3): p. 373-6.

98. Gaidano, G., L. Pasqualucci, D. Capello, E. Berra, C. Deambrogi, D. Rossi, L. Maria Larocca, A. Gloghini, A. Carbone, and R. Dalla-Favera, *Aberrant somatic hypermutation in multiple subtypes of AIDS-associated non-Hodgkin lymphoma.* Blood, 2003. **102**(5): p. 1833-41.

99. Montesinos-Rongen, M., D. Van Roost, C. Schaller, O.D. Wiestler, and M. Deckert, *Primary diffuse large B-cell lymphomas of the central nervous system are targeted by aberrant somatic hypermutation.* Blood, 2004. **103**(5): p. 1869-75.

100. Deutsch, A.J., M. Fruhwirth, A. Aigelsreiter, L. Cerroni, and P. Neumeister, *Primary cutaneous marginal zone B-cell lymphomas are targeted by aberrant somatic hypermutation.* J Invest Dermatol, 2009. **129**(2): p. 476-9.

101. Deutsch, A.J., A. Aigelsreiter, P.B. Staber, A. Beham, W. Linkesch, C. Guelly, R.I. Brezinschek, M. Fruhwirth, W. Emberger, M. Buettner, C. Beham-Schmid, and P. Neumeister, *MALT lymphoma and extranodal diffuse large B-cell lymphoma are targeted by aberrant somatic hypermutation.* Blood, 2007. **109**(8): p. 3500-4.

102. Reiniger, L., C. Bodor, A. Bognar, Z. Balogh, J. Csomor, A. Szepesi, L. Kopper, and A. Matolcsy, *Richter's and prolymphocytic transformation of chronic lymphocytic leukemia are associated with high mRNA expression of activation-*

induced cytidine deaminase and aberrant somatic hypermutation. Leukemia, 2006. **20**(6): p. 1089-95.

103. Rossi, D., E. Berra, M. Cerri, C. Deambrogi, C. Barbieri, S. Franceschetti, M. Lunghi, A. Conconi, M. Paulli, A. Matolcsy, L. Pasqualucci, D. Capello, and G. Gaidano, *Aberrant somatic hypermutation in transformation of follicular lymphoma and chronic lymphocytic leukemia to diffuse large B-cell lymphoma.* Haematologica, 2006. **91**(10): p. 1405-9.
104. Rabbitts, T.H., R. Baer, M. Davis, A. Forster, P.H. Hamlyn, and S. Malcolm, *The c-myc gene paradox in Burkitt's lymphoma chromosomal translocation.* Curr Top Microbiol Immunol, 1984. **113**: p. 166-71.
105. Rabbitts, T.H., P.H. Hamlyn, and R. Baer, *Altered nucleotide sequences of a translocated c-myc gene in Burkitt lymphoma.* Nature, 1983. **306**(5945): p. 760-5.
106. Cuypers, H.T., G. Selten, W. Quint, M. Zijlstra, E.R. Maandag, W. Boelens, P. van Wezenbeek, C. Melief, and A. Berns, *Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region.* Cell, 1984. **37**(1): p. 141-50.
107. Rogozin, I.B. and N.A. Kolchanov, *Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis.* Biochim Biophys Acta, 1992. **1171**(1): p. 11-8.
108. Rogozin, I.B., V.V. Solovyov, and N.A. Kolchanov, *Somatic hypermutagenesis in immunoglobulin genes. I. Correlation between somatic mutations and repeats. Somatic mutation properties and clonal selection.* Biochim Biophys Acta, 1991. **1089**(2): p. 175-82.
109. Zan, H., A. Cerutti, P. Dramitinos, A. Schaffer, Z. Li, and P. Casali, *Induction of Ig somatic hypermutation and class switching in a human monoclonal IgM+ IgD+ B cell line in vitro: definition of the requirements and modalities of hypermutation.* J Immunol, 1999. **162**(6): p. 3437-47.
110. Muramatsu, M., V.S. Sankaranand, S. Anant, M. Sugai, K. Kinoshita, N.O. Davidson, and T. Honjo, *Specific expression of activation-induced cytidine*

deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. J Biol Chem, 1999. **274**(26): p. 18470-6.

111. Longerich, S., U. Basu, F. Alt, and U. Storb, *AID in somatic hypermutation and class switch recombination.* Curr Opin Immunol, 2006. **18**(2): p. 164-74.
112. Bransteitter, R., P. Pham, M.D. Scharff, and M.F. Goodman, *Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 4102-7.
113. Petersen-Mahrt, S.K., R.S. Harris, and M.S. Neuberger, *AID mutates E. coli suggesting a DNA deamination mechanism for antibody diversification.* Nature, 2002. **418**(6893): p. 99-103.
114. Luo, Z., D. Ronai, and M.D. Scharff, *The role of activation-induced cytidine deaminase in antibody diversification, immunodeficiency, and B-cell malignancies.* J Allergy Clin Immunol, 2004. **114**(4): p. 726-35; quiz 736.
115. Dorsett, Y., D.F. Robbiani, M. Jankovic, B. Reina-San-Martin, T.R. Eisenreich, and M.C. Nussenzweig, *A role for AID in chromosome translocations between c-myc and the IgH variable region.* J Exp Med, 2007. **204**(9): p. 2225-32.
116. Lossos, I.S., R. Levy, and A.A. Alizadeh, *AID is expressed in germinal center B-cell-like and activated B-cell-like diffuse large-cell lymphomas and is not correlated with intraclonal heterogeneity.* Leukemia, 2004. **18**(11): p. 1775-9.
117. Greeve, J., A. Philipsen, K. Krause, W. Klapper, K. Heidorn, B.E. Castle, J. Janda, K.B. Marcu, and R. Parwaresch, *Expression of activation-induced cytidine deaminase in human B-cell non-Hodgkin lymphomas.* Blood, 2003. **101**(9): p. 3574-80.
118. Hardianti, M.S., E. Tatsumi, M. Syampurnawati, K. Furuta, K. Saigo, Y. Nakamachi, S. Kumagai, H. Ohno, S. Tanabe, M. Uchida, and N. Yasuda, *Activation-induced cytidine deaminase expression in follicular lymphoma: association between AID expression and ongoing mutation in FL.* Leukemia, 2004. **18**(4): p. 826-31.

119. Miller, S.A., D.D. Dykes, and H.F. Polesky, *A simple salting out procedure for extracting DNA from human nucleated cells.* Nucleic Acids Res, 1988. **16**(3): p. 1215.

120. Bahler, D.W., M.J. Campbell, S. Hart, R.A. Miller, S. Levy, and R. Levy, *Ig VH gene expression among human follicular lymphomas.* Blood, 1991. **78**(6): p. 1561-8.

121. Deane, M. and J.D. Norton, *Immunoglobulin heavy chain variable region family usage is independent of tumor cell phenotype in human B lineage leukemias.* Eur J Immunol, 1990. **20**(10): p. 2209-17.

122. Chang, B.p. and P.p. Casali, *The CDR1 sequences of a major proportion of human germline Ig VH genes are\par inherently susceptible to amino acid replacement.\par.* Immunol Today\par, 1994. **15\par**(8\par): p. 367-73\par.

123. Lossos, I.S., R. Tibshirani, B. Narasimhan, and R. Levy, *The inference of antigen selection on Ig genes.* J Immunol, 2000. **165**(9): p. 5122-6.

124. Bosga-Bouwer, A.G., G.W. van Imhoff, R. Boonstra, A. van der Veen, E. Haralambieva, A. van den Berg, B. de Jong, V. Krause, M.C. Palmer, R. Coupland, P.M. Kluin, E. van den Berg, and S. Poppema, *Follicular lymphoma grade 3B includes 3 cytogenetically defined subgroups with primary t(14;18), 3q27, or other translocations: t(14;18) and 3q27 are mutually exclusive.* Blood, 2003. **101**(3): p. 1149-54.

125. Godon, A., A. Moreau, P. Talmant, L. Baranger-Papot, F. Genevieve, N. Milpied, M. Zandecki, and H. Avet-Loiseau, *Is t(14;18)(q32;q21) a constant finding in follicular lymphoma? An interphase FISH study on 63 patients.* Leukemia, 2003. **17**(1): p. 255-9.

126. Guo, Y., K. Karube, R. Kawano, T. Yamaguchi, J. Suzumiya, G.S. Huang, and K. Ohshima, *Low-grade follicular lymphoma with t(14;18) presents a homogeneous disease entity otherwise the rest comprises minor groups of heterogeneous disease entities with Bcl2 amplification, Bcl6 translocation or other gene aberrances.* Leukemia, 2005. **19**(6): p. 1058-63.

127. Horsman, D.E., I. Okamoto, O. Ludkovski, N. Le, L. Harder, S. Gesk, R. Siebert, M. Chhanabhai, L. Sehn, J.M. Connors, and R.D. Gascoyne, *Follicular lymphoma lacking the t(14;18)(q32;q21): identification of two disease subtypes.* Br J Haematol, 2003. **120**(3): p. 424-33.

128. Bosga-Bouwer, A.G., A. van den Berg, E. Haralambieva, D. de Jong, R. Boonstra, P. Kluin, E. van den Berg, and S. Poppema, *Molecular, cytogenetic, and immunophenotypic characterization of follicular lymphoma grade 3B; a separate entity or part of the spectrum of diffuse large B-cell lymphoma or follicular lymphoma?* Hum Pathol, 2006. **37**(5): p. 528-33.

129. Guo, Y., K. Karube, R. Kawano, J. Suzumiya, M. Takeshita, M. Kikuchi, G.S. Huang, Q. Li, and K. Ohshima, *Bcl2-negative follicular lymphomas frequently have Bcl6 translocation and/or Bcl6 or p53 expression.* Pathol Int, 2007. **57**(3): p. 148-52.

130. Jardin, F., P. Gaulard, G. Buchonnet, N. Contentin, S. Lepretre, P. Lenain, A. Stamatoullas, J.M. Picquenot, C. Duval, F. Parmentier, H. Tilly, and C. Bastard, *Follicular lymphoma without t(14;18) and with BCL-6 rearrangement: a lymphoma subtype with distinct pathological, molecular and clinical characteristics.* Leukemia, 2002. **16**(11): p. 2309-17.

131. Tagawa, H., K. Karube, Y. Guo, M. Takeshita, M. Kikuchi, Y. Morishima, S. Nakamura, K. Ohshima, and M. Seto, *Trisomy 3 is a specific genomic aberration of t(14;18) negative follicular lymphoma.* Leukemia, 2007. **21**(12): p. 2549-51.

132. Gu, K., K. Fu, S. Jain, Z. Liu, J. Iqbal, M. Li, W.G. Sanger, D.D. Weisenburger, T.C. Greiner, P. Aoun, B.J. Dave, and W.C. Chan, *t(14;18)-negative follicular lymphomas are associated with a high frequency of BCL6 rearrangement at the alternative breakpoint region.* Mod Pathol, 2009. **22**(9): p. 1251-7.

133. Karube, K., Y. Guo, J. Suzumiya, Y. Sugita, Y. Nomura, K. Yamamoto, K. Shimizu, S. Yoshida, H. Komatani, M. Takeshita, M. Kikuchi, N. Nakamura, O. Takasu, F. Arakawa, H. Tagawa, M. Seto, and K. Ohshima, *CD10-MUM1+ follicular lymphoma lacks BCL2 gene translocation and shows characteristic biologic and clinical features.* Blood, 2007. **109**(7): p. 3076-9.

134. Piccaluga, P.P., A. Califano, U. Klein, C. Agostinelli, B. Bellosillo, E. Gimeno, S. Serrano, F. Sole, Y. Zang, B. Falini, P.L. Zinzani, and S.A. Pileri, *Gene expression analysis provides a potential rationale for revising the histological grading of follicular lymphomas.* Haematologica, 2008. **93**(7): p. 1033-8.

135. Zelenetz, A.D., T.T. Chen, and R. Levy, *Clonal expansion in follicular lymphoma occurs subsequent to antigenic selection.* J Exp Med, 1992. **176**(4): p. 1137-48.

136. Zha, H., M. Raffeld, L. Charboneau, S. Pittaluga, L.W. Kwak, E. Petricoin, 3rd, L.A. Liotta, and E.S. Jaffe, *Similarities of prosurvival signals in Bcl-2-positive and Bcl-2-negative follicular lymphomas identified by reverse phase protein microarray.* Lab Invest, 2004. **84**(2): p. 235-44.

137. Rajnai, H., C. Bodor, Z. Balogh, E. Gagyi, J. Csomor, T. Krenacs, E. Toth, and A. Matolcsy, *Impact of the reactive microenvironment on the bone marrow involvement of follicular lymphoma.* Histopathology, 2012. **60**(6B): p. E66-E75.

138. Leich, E., A. Zamo, H. Horn, E. Haralambieva, B. Puppe, R.D. Gascoyne, W.C. Chan, R.M. Braziel, L.M. Rimsza, D.D. Weisenburger, J. Delabie, E.S. Jaffe, J. Fitzgibbon, L.M. Staudt, H.K. Mueller-Hermelink, M. Calaminici, E. Campo, G. Ott, L. Hernandez, and A. Rosenwald, *MicroRNA profiles of t(14;18)-negative follicular lymphoma support a late germinal center B-cell phenotype.* Blood, 2011. **118**(20): p. 5550-8.

139. Bodor, C., C. O'Riain, D. Wrench, J. Matthews, S. Iyengar, H. Tayyib, M. Calaminici, A. Clear, S. Iqbal, H. Quentmeier, H.G. Drexler, S. Montoto, A.T. Lister, J.G. Gribben, A. Matolcsy, and J. Fitzgibbon, *EZH2 Y641 mutations in follicular lymphoma.* Leukemia, 2011. **25**(4): p. 726-9.

140. Morin, R.D., N.A. Johnson, T.M. Severson, A.J. Mungall, J. An, R. Goya, J.E. Paul, M. Boyle, B.W. Woolcock, F. Kuchenbauer, D. Yap, R.K. Humphries, O.L. Griffith, S. Shah, H. Zhu, M. Kimbara, P. Shashkin, J.F. Charlot, M. Tcherpakov, R. Corbett, A. Tam, R. Varhol, D. Smailus, M. Moksa, Y. Zhao, A. Delaney, H. Qian, I. Birol, J. Schein, R. Moore, R. Holt, D.E. Horsman, J.M. Connors, S. Jones, S. Aparicio, M. Hirst, R.D. Gascoyne, and M.A. Marra, *Somatic mutations*

altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nat Genet, 2010. **42**(2): p. 181-5.

DOI:10.14753/SE.2012.1744

XI. THE CANDIDATE'S PUBLICATION LIST

Articles related to the subject of the dissertation:

1. Rajnai H, Bödör Cs, Balogh Zs, **Gagyi É**, Csomor J, Krenács T, Tóth E, Matolcsy A. Impact of the reactive microenvironment on the bone marrow involvement of follicular lymphoma. *Histopathology*, 2012. 60(6B): p. E66-E75.
IF: 3.569

2. **Gagyi É**, Balogh Zs, Bödör Cs, Timár B, Reiniger L, Deák L, Csomor J, Csernus B, Szepesi Á, Matolcsy A. Somatic hypermutation of IgV_H genes and aberrant somatic hypermutation in follicular lymphoma without *BCL-2* gene rearrangement and expression. *Haematologica*, 2008;93:1822-1828.
IF: 5.978

3. Balogh Zs, Reiniger L, Deák L, Bödör Cs, Csomor J, Szepesi Á, **Gagyi É**, Kopper L, Matolcsy A. IgV_H gene mutation status and genomic imbalances in chronic lymphocytic leukemia with increased prolymphocytes (CLL/PL). *Hematological Oncology*, 2007;25: 90-95.
IF: 2.180

Articles in different subject:

1. Castellanos K.J, **Gagyi É**, Kormos B, Valyi-Nagy K, Voros A, Shukla D, Horvath S, Slavin K.V, Valyi-Nagy T. Increased axonal expression of nectin-1 in multiple sclerosis plaques. *Neurological Sciences*, 2012, DOI: 10.1007/s10072-012-1026-9. **IF: 1.22**

2. **Gagyi É**, Kormos B, Castellanos K.J, Valyi-Nagy K, Korneff D, Lopresti P, Woltjer R, Valyi-Nagy T. Decreased Oligodendrocyte Nuclear Diameter in

Alzheimer's Disease and Lewy Body Dementia. *Brain Pathology*, 2012, DOI: 10.1111/j.1750-3639.2012.00595.x. **IF: 4.741**

3. Balogh Zs, Reiniger L, Rajnai H, Csomor J, Szepesi A, Balogh A, Deák L, **Gagyi É**, Bödör Cs, Matolcsy A. High rate of neoplastic cells with genetic abnormalities in proliferation centers of chronic lymphocytic leukemia. *Leukemia & Lymphoma*, 2011;52(6):1080-4. **IF: 2.492**

4. Dosa S, Castellanos K.J, Bacsa S, **Gagyi É**, Kovacs SK, Valyi-Nagy K, Shukla D, Dermody T.S, Valyi-Nagy T. Chronic progressive deficits in neuron size, density and number in the trigeminal ganglia of mice latently infected with herpes simplex virus. *Brain Pathology*, 2011;21(5):583-93.

IF: 4.741

5. **Gagyi É**, Horváth E, Bödör Cs, Timár B, Matolcsy A, Pávai Z. Prognostic significance and detection of the Internal Tandem Duplication of the FLT3 gene in acute myeloid leukemia. *Romanian Journal of Morphology and Embryology*, 2006;47:331-337.

Book chapter:

Valyi-Nagy K, Voros A, **Gagyi É**, Valyi-Nagy T. (2011) Increased resistance of vasculogenic mimicry-forming uveal melanoma cells against cytotoxic agents in three-dimensional cultures. In: Research on Melanoma - A Glimpse into Current Directions and Future Trends. Murph Mandi (Ed.), InTech, Vienna, Austria. ISBN: 978-953-307-293-7.

DOI:10.14753/SE.2012.1744

XIII. ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to all, whose generous help and support made it possible to complete this work. First of all I would like to thank my parents, family and friends for their patience and support, unwavering love and encouragement, which was essential for the completion of my work.

I would like to thank my tutor, **Prof. Dr. András Matolcsy** for the opportunity to perform my PhD work in his Department as part of his research team. I am deeply grateful for his trust, continuous support, scientific advice, understanding and encouragement during both my research work and my studies.

I would like to thank **Prof. Dr. László Kopper**, who has provided me absolute support to progress in scientific research.

I am grateful to **Dr. Tibor Krenács** for his detailed attention in reviewing my thesis and all his help, valuable comments and useful advices.

I would like to thank **Dr. Judit Csomor, Dr. Ágota Szepesi, Dr. Emőke Horváth** and **Dr. Erika Tóth** for their support, advice, encouragement and kindness.

I am deeply grateful to my colleagues and friends for being supportive and giving valuable help and useful advices for my work, for the pleasant work environment and great companionship: **Dr. Csaba Bödör**, **Dr. Zsófia Balogh**, **Dr. Lilla Reiniger**, **Dr. Botond Timár**, **Dr. Hajnalka Rajnai**, **Dr. Balázs Csernus**, **Dr. Ágnes Bognár**, **Dr. Kinga Szurián, Dr. Gábor Barna**, **Dr. Linda Moskovszky** and **Dr. Katalin Dezső**.

I wish to express my gratitude to the assistants whom I worked together with: **Adrienne Bárányné Pallag**, **Anikó Lengyel**, **Linda Deák, Bernadett Budai, Orsolya Muhari**, **Orsolya Szabó** and **Ildikó Szabó** for their continuous help and support, for the pleasant work atmosphere and their friendship.

I am grateful to **Prof. Dr. Ilona Kovalszky** and **Dr. Tibor Füle** for the chance to work in their lab and for their help in my work.

I would like to thank **Cecília Laczik, Ilona Szilágyi and Erzsébet Tegzes** for their patience, help and support whenever I needed.