Mutational patterns of IGHV and BCL6 genes in B-cell Non-Hodgkin lymphomas

PhD thesis

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LIST OF ABBREVIATIONS

ABC	- activated B-cell like
AID	- activation-induced cytidine deaminase
AIDS	- acquired immunodeficiency syndrome
ALL	- acute lymphoblastic leukaemia
APC	- antigen presenting cell
ASHM	- aberrant somatic hypermutation
ATM	- ATM serine/threonine kinase,
BCL6	- B-cell lymphoma 6
BCR	- B-cell receptor
BLIMP1	- protein that in humans is encoded by the PRDM1 gene
BM	- bone marrow
BTB/POZ	- BTB (for BR-C, ttk and bab) and POZ (for Pox virus and Zinc finger) domain is present near the N-terminus of a fraction of zinc finger proteins
CDR	- complementary determining region
CLL	- chronic lymphocytic leukaemia
C segment	- constant segment
DLBCL	- diffuse large B-cell lymphoma
DNA	- deoxyribonucleic acid
D segment	- diversity segment
EMBL	- European Molecular Biology Laboratory
FDC	- follicular dendritic cell
FL	- follicular lymphoma
FR	- framework
GC	- germinal center
Ig	- immunoglobulin

IgH	- immunohhlobulin heavy chain
IgL	- immunoglobulin light chain
IGHV	- immunoglobulin heavy chain variable region
ISFL	- in situ follicular lymphoma
J segment	- joining serment
LN	- lymph node
MCL	- mantle cell lymphoma
MHC	- major histocompatibility complex
MZL	- marginal zone lymphoma
NCBI	- National Center for Biotechnology Information
NHL	- non-Hodgkin lymphoma
PCR	- polymerase chain reaction
PMBL	- primary mediastinal B-cell lymphoma
SHM	- somatic hypermutation
SNP	- single nucleotide polymorphism
SSCP	- single strand conformation polymorphism
TSS	- transcription start site
UTR	- untranslated region
VH	- heavy chain variable gene
V segment	- variable segment
WHO	- World Health Organization

1. INTRODUCTION

B-cell non-Hodgkin lymphomas are a heterogeneous group of malignant disorders characterized by variable morphologic, immunophenotypic, genetic, and clinical features. The malignant lymphocytes in each of the entities are considered to be clonal descendants of a transformed progenitor cell arrested at various stages of lymphocyte differentiation. A highly characteristic and specific feature of physiological B-lymphocyte maturation is the rearrangement and affinity maturation of the immunoglobulin (Ig) genes ultimately producing a functionally active, antigen specific and broad B-cell receptor (BCR) repertoire. Molecular analysis of the Ig gene rearrangement is able to deliver valuable insight into the character of the different lymphoproliferative entities. At the most basic level, it provides genetic support for the monoclonal nature of the diseases by demonstrating uniform Ig gene rearrangement in all tumor cells. The in depth analysis of the somatic mutation pattern of the Ig heavy-chain gene variable regions (IGHV) provides valuable information about exposure to antigen selection in a suitable microenvironment such as the germinal center (GC). The evaluation of the mutational status of the IGHV gene enables us to establish the cellular origin of the malignant clone with respect to the germinal center (pre-follicular, follicular or post-follicular) supplying additional clues for lymphoma classification. This information can also be used to determine the clonal composition/relatedness of the malignant cell population allowing us to monitor the clonal evolution and disease progression. In a similar fashion, the study of aberrant somatic hypermutation, targeting key regulatory genes of lymphocyte maturation outside of the IGHV loci, can also be related to high-grade transformation and progression.

1.1 The process of physiological IgH gene rearrangement and somatic mutations

1.1.1 Early stage: Bone marrow – IgH rearrangement

B-lymphocytes are generated in the bone marrow from stem cells, which produce about 60 billion new B cells every day (1). In the first phase of development the immature B-cell precursors acquire functional antigen receptors through rearrangements of the germline immunoglobulin genes. The complete process to develop a mature B-lymphocyte takes about 1-2 weeks. The result will be a mature B cell expressing a surface B-cell receptor assembled from immunoglobulin heavy (IgH) and light chains (2).

The genomic organization of immunoglobulin genes is quite unique and differs from all other genes (3). In all cells, except B cells, the immunoglobulin genes are found in a non-functional, fragmented form the so called germline configuration that cannot be expressed. The gene segments, representing alternative versions of different parts of the immunoglobulin heavy and light chains coding for the B-cell receptors, are sequentially arranged and can only be successfully expressed following their genetic rearrangement, through a process unique to B-cells. The gene rearrangements take place during early B-cell development in the bone marrow. In case these rearrangements are successful, complete, heavy and light chains are produced, and membrane-bound immunoglobulins, better known as the B-cell receptors are expressed on the cell surface (1).

In humans, the immunoglobulin genes are located on three different chromosomes: the heavy-chain locus is on chromosome 14, the κ light-chain locus is found on chromosome 2, while chromosome 22 holds the λ light-chain locus (4). Each heavy and light chain is assembled from gene segments composed of a leader peptide (L), a V region (V), and a constant region (C). In essence, the V regions represent highly variable sequences that are responsible for antigen recognition, while the evolutionally more conserved C regions mediate the effector functions. The V regions are encoded by two gene segments (V and J) in case of the light chains, and three gene segments (V, D and J) in the heavy chains. Each of these segments are randomly selected from an array of similar, alternative gene sequences

through a recombination process, and after elimination of the interposed introns, they are joined to produce an exon that can be transcribed (3). The V regions of the light-chains consist of only two segments V (variable) and J (joining). The heavy-chain locus includes an additional set of diversity (D) gene segments that lies between the arrays of V and J gene segments. Thus the V region of a heavy-chain is encoded by a unique combination of one V, one D, and one J gene segment (5).

During B-cell development, the arrays of V, D, and J segments are cut and re-spliced by a process called DNA recombination. This process brings together a single gene segment of each type to form a DNA sequence encoding the V region of an immunoglobulin chain. For heavy chains, a two-step recombination is needed, the first to join a D and a J segment, and the second to join the combined DJ segment to a V segment. (**Figure 1.**)

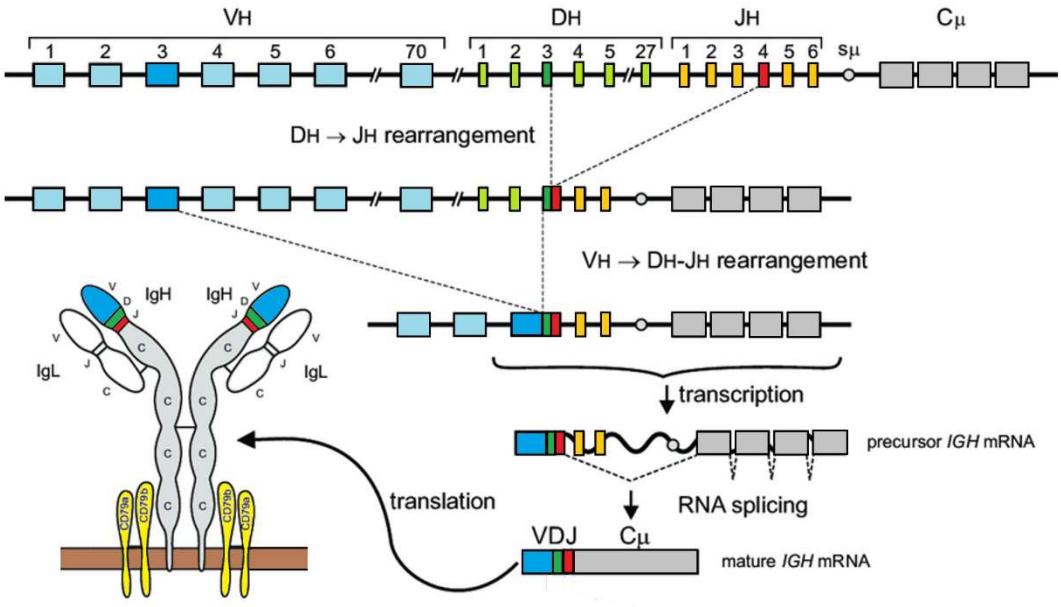

Figure 1 **.The process of human IGH gene rearrangement**. Schematic representation of the human germline immunoglobulin heavy chain (IGH) locus on chromosome 14q32 indicating the presence of 70 VH genes, 27 DH genes, 6 JH genes. Following RAG-mediated random recombination of alternative gene segments each pre B-cell generates a functional B-cell receptor consisting of a unique combination of VDJ genes joined with Cμ constant gene. The process takes place in the bone marrow.

In each case, the particular V, D, and J gene segments that are joined together are selected at random. Because of the multiple gene segments of each type, numerous different combinations of V, D, and J gene segments are possible. Thus, the random gene rearrangement process generates many different V-region sequences in the B-cell population. This is just one of the factors contributing to the diversity of immunoglobulin V regions (5). Once the VDJ rearrangement is complete the gene is transcribed through the nearest C gene segment (μ) as a complete μ heavy-chains located in the cytoplasm of pre-B-cells. Rearrangement of the light-chain genes follow. The κ light-chain genes are the first to rearrange, and only if those rearrangements fail to make a viable κ chain are the λ light-chain genes rearranged. Following a successful light-chain rearrangement, a light-chain protein is synthesized and assembled in the endoplasmic reticulum with the μ heavy-chains to form membrane-bound IgM (5).

1.1.2 Late stage: IgH somatic hypermutation in peripheral lymphoid organs

Following successful Ig gene rearrangement, the naïve B-cells expressing a membrane bound B-cell receptor (IgM) migrate to the peripheral lymphoid organs for "fine-tuning" their antigen specificity. The germinal center (GC) is the histological structure dedicated to the generation and selection of B-cells that produce high-affinity antibodies through a process called affinity maturation (6). The procedure is aided by antigen presenting cells including the follicular dendritic cells (FDC) and follicular helper T-cells ($T\text{-}_{FH}$) localized within the germinal centers. The first step of the process involves unique genetic modifications of the complementary determining regions (CDR – section of the V region directly binding the antigen) of the immunoglobulin genes by introducing random point mutations through somatic hypermutation. The average mutation rate in this supportive millieau is about 1 million times higher than elsewhere resulting in a mutational frequency of 10^{-3} point mutations per base pair in the variable region during each cycle (7). The process is catalyzed by an enzyme called activation-induced cytidine deaminase (AID) responsible for generating these mutations by converting cytidine to uridine to initiate the hypermutation

process. Error-prone versions of DNA repair are believed to then convert these lesions into a diverse spectrum of point mutations. The second step of affinity maturation is clonal selection, where only those B-cell clones that were able to improve the binding affinity to their antigens are let to survive. Several cycles of repeated exposures to the same antigen will produce antibodies of successively greater affinities. The combined result of genetic rearrangement and somatic hypermutation is able to generate an estimated 10^{15}–10^{18} possible unique B-cell clones to help in the fight against all possible intruding antigens (8). *(***Figure 2***.)*

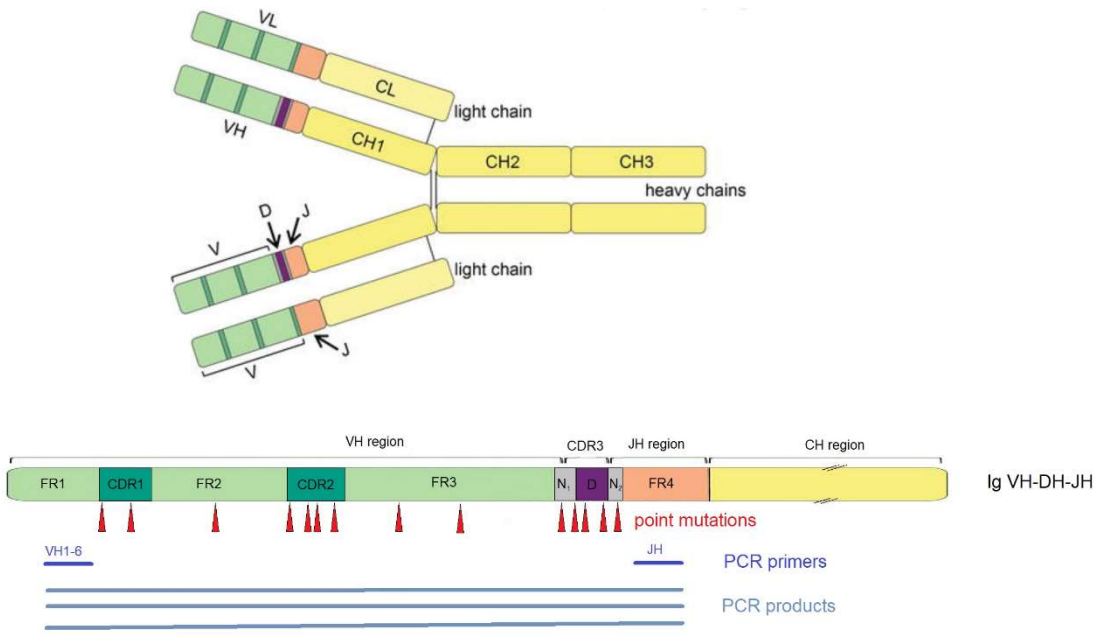

Figure 2. **Somatic hypermutation of IgH gene**. The VH regions of the IgH genes undergo AID mediated somatic hypermutation during B-cell transit through the germinal centers. Random point mutations are introduced into the FR and CDR regions of the IGVH gene during affinity maturation. The lower part of the figure shows the binding site of the forward (VH1-6) and reverse (JH) primers used to amplify the VH region. The forward primers attach to FR1, while the reverse primer attach to FR4.

1.2 Cellular origin of lymphoproliferative malignancies

Similarly, to solid tumors, the cell of origin in lymphoproliferative malignancies holds major clinical importance regarding prognosis and therapeutic strategies (9-11). By

their nature, lymphoid cells continuously recirculate between the different lymphoid organs, so in contrast to solid tumors, the cellular origin of lymphoproliferative disease does not necessarily refer to a specific organ or location, rather it is associated with the stages of cellular differentiation. Each stage of differentiation necessitates elaborate alterations of gene expression that often leads to phenotypical alterations, and may or may not be accompanied by morphological changes. In addition to transcriptional and translational changes, certain steps of B-cell maturation require genetic alterations of the DNA sequence in form of genomic rearrangements and somatic mutations involving the immunoglobulin heavy chain gene (IgH) (5).

Molecular analysis of immunoglobulin heavy chain mutational status including the V-D-J rearrangement of the immunoglobulin heavy chain gene, class switch recombination together with the presence and distribution pattern of somatic mutation in the V-regions delivers valuable information about the stage of lymphoid differentiation the malignant transformation had occurred (12, 13). Based on the combination of these clonal rearrangements and mutations we can track the stage of B-cell differentiation where the malignant transformation had occurred. (**Figure 3.)** With respect the stage of development occurring in the germinal centers, the various B-cell NHL entities can be classified into prefollicular, follicular and postfollicular origin. The prefollicular stage is characterized by germline configuration of the IgH gene found in precursor B-cell entities, or rearranged, but unmutated IGHV sequences, for entities originating from naïve, antigen inexperienced B-cells. All mature B-cell non-Hodgkin lymphomas harbor rearranged IgH sequences. A mutated IgH gene indicates an antigen experienced B-cell that has transited through the GC seen in the majority of mature B-cell lymphomas. The entities originating directly from the germinal center stage of development, regarded as of follicular origin, display significant intraclonal heterogeneity (same IGHV rearrangement, but variable V-region somatic mutations) indicating presence active mutational machinery leading to continuously evolving (ongoing) V-region mutations (14). The postgerminal, mature B-cell lymphomas contain rearranged, mutated IGHV sequences without evolving intraclonal diversity since the mutational machinery is forced to shut-down when the B-cell exits the germinal center.

Further, in-depth analysis of the pattern and distribution of somatic mutations in the V-regions allows the identification of genetic footprint of antigen selection (antigen driven affinity maturation) in contrast to just randomly occurring mutations (7).

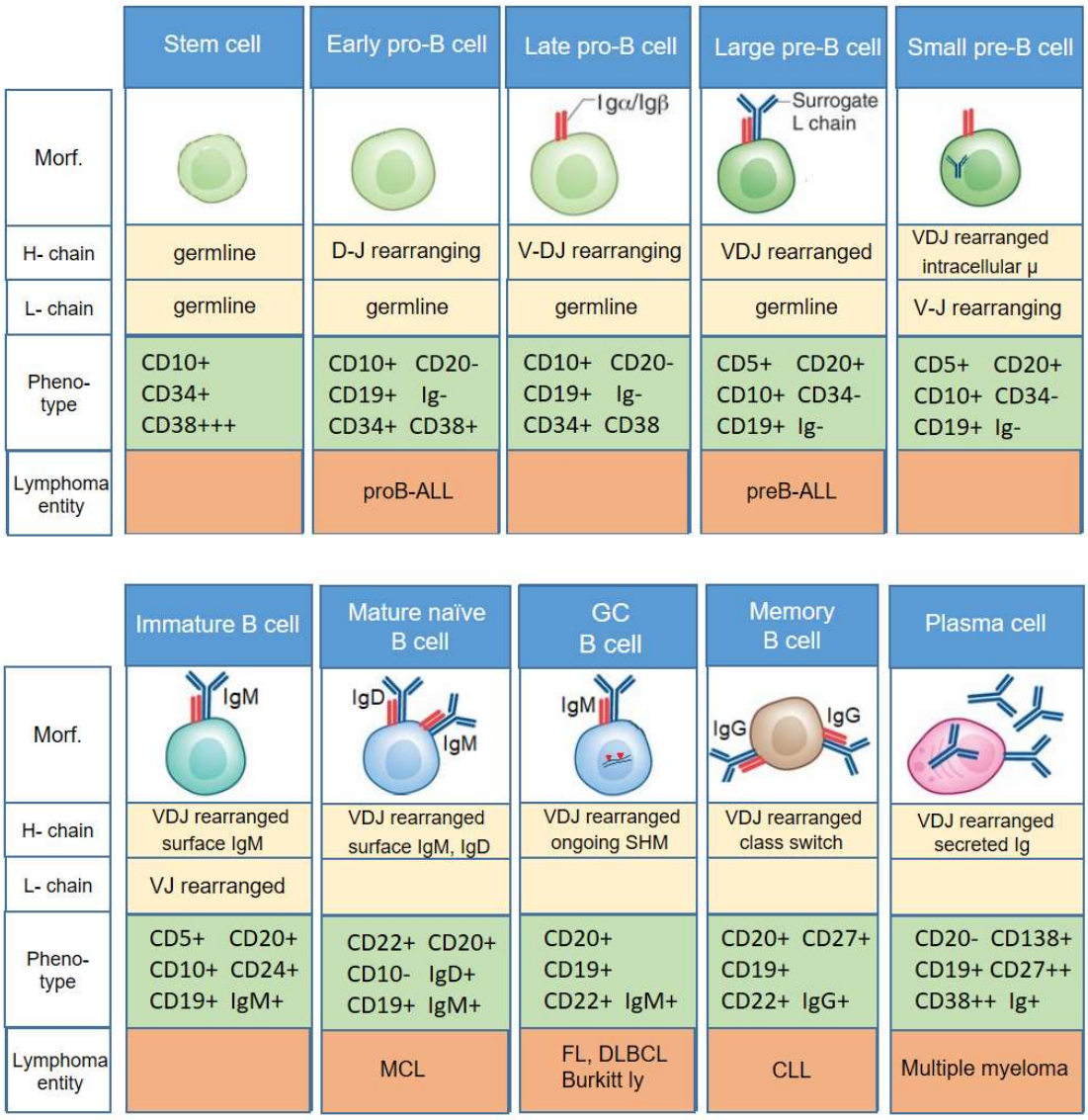

Figure 3. **Alterations of the IGH gene sequence and BCR expression during B-cell differentiation.** Figure shows the progressive alterations affecting the IgH and IgL gene sequences, as well as the changes of B-cell receptor and antigen expression characterizing each stage of B-cell development. The orange fields display the most frequent NHL entities originating from the particular developmental stage.

The routine diagnostic procedure for determination of cellular origin of a lymphoma entity is usually based on easily accessible features like clinical appearance, morphology, and immunophenotype. In a significant fraction of the cases however, these basic diagnostic approaches provide equivocal results, since the malignant transformation often leads to complex derailment of the normal maturation process resulting in unusual combinations of antigen expression that is difficult to interpret. These cases require a deeper level of investigation aimed at uncovering the underlying genetic alterations with sophisticated methods including gene expression profiling, interphase cytogenetics and mutational profiling of the immunoglobulin and other key regulatory genes. As outlined above, the genetic analysis of immunoglobulin heavy chain mutational status with respect to the V-D-J rearrangement and presence of somatic mutation of the V-regions delivers valuable information about the stage of lymphoid maturation the malignant transformation had occurred. The vast majority of mature B-cell NHL entities, originate from stages of maturation revolving around the germinal center, were the B-cells are not only subjected to intense proliferation, but also to genetic remodeling including somatic hypermutation and class-switch recombination. Naturally, potentially transforming mutations occur with a lot higher frequency in association with these stages of development (15). **Figure 4.** on the following page depicts the link between the currently recognized WHO B-NHL entities and the stages of B-cell development with respect to the germinal center.

1.3 IGHV mutational analysis for study of clonal evolution and progression

As the combination of Ig rearrangements and somatic mutations are unique and highly specific signatures of an individual B-cell clone, molecular analysis of these alterations

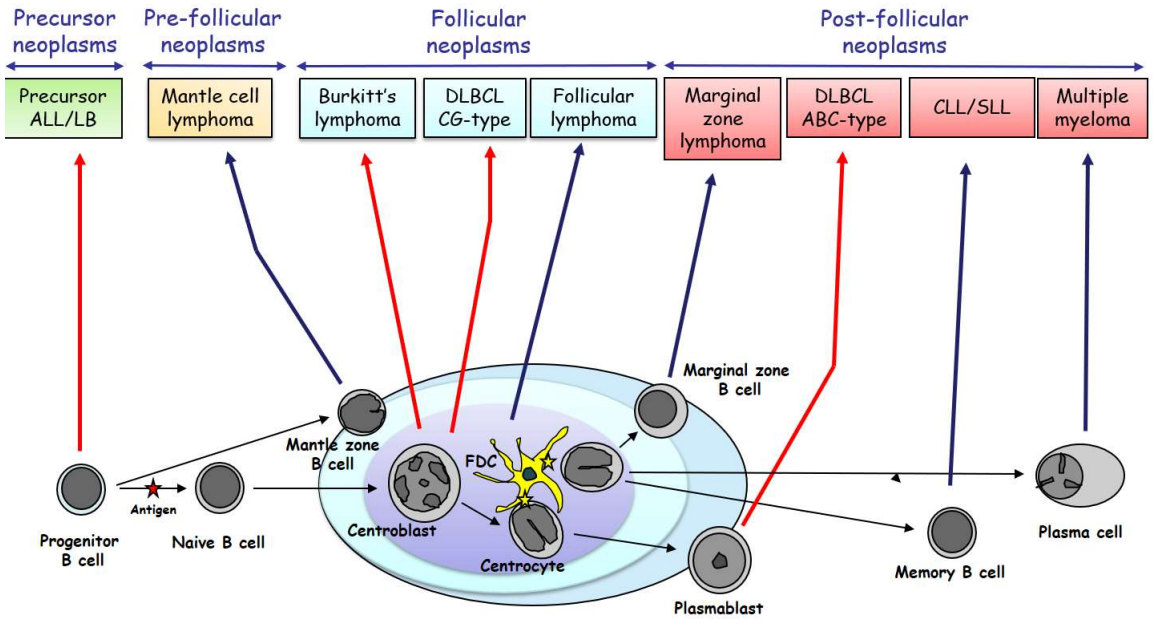

Figure 4. **Cellular origin of B-NHL**. Most of the currently recognized entities in the WHO classification of lymphoproliferative disease can be related to the clonal expansion of B-lymphocytes halted at various stages of their physiological maturation. Each differentiation stage from naïve antigen inexperienced B-cells to memory B-cells and plasma cells can be linked to a mature B-NHL entity originating from various compartments of the B-cell follicle.

allows us to collect information about the clonal composition and clonal relatedness (clonal evolution) of the neoplastic population (13). Several different mutational patterns are recognized. The lack IGHV somatic mutations denotes an antigen inexperienced B-cell population compatible with precursor B-cell diseases (such as B-ALL). The IGHV mutation positive clonal B-cell populations can be further subdivided based on the homogeneity or heterogeneity of the mutated subclones. A tumor composed of genetically homogeneous population of IGHV mutated clonal B-cells indicates a lymphoma with postgerminal center origin, that has transited through the germinal center, and already completed the process of somatic hypermutation. On the other hand, a tumor with heterogeneous clonal populations differing in IGHV somatic mutations point toward the ongoing nature of somatic hypermutation, outlining a cell population that has frozen at the germinal center stage of maturation. These malignancies either directly originate from the germinal center, or from a GC equivalent milieu where all necessary components of the somatic mutational machinery are readily available. Additional genetic evidence for antigen driven clonal selection of the B-cell clones can be gathered by comparative analysis of the number of replacement

mutations affecting the complementary determining (CDR) and framework (FR) regions of the IGHV gene segments.

In case of an intraclonally heterogeneous tumor cell populations, pattern of shared and unique mutations allows the construction of genealogical trees reflecting the clonal evolution of the neoplastic cells. In these genealogical trees the individual subclones differ from the common progenitor by progressively accumulated nucleotide changes assuming that shared mutations represent single events and not independent mutations. Careful analysis of the sequence polymorphism allows the identification of the putative clonal progenitor.

1.4 BCL6 gene and aberrant somatic hypermutation

The initiation of the GC reaction is dependent on the induction of several transcriptional modulators, including BCL6. This gene is frequently affected by genetic alterations including mutations and translocations in various B-cell lymphoma entities. BCL6 is a 95-kD nuclear phosphoprotein belonging to the BTB/POZ zinc finger family of transcription factors. It has been suggested that BCL6 is important in the repression of genes involved in the control of lymphocyte activation, differentiation, and apoptosis within the germinal center, and that its down-regulation is necessary for normal B-cells to exit the germinal center (16). BCL6 physiologically represses BLIMP1 a transcription factor that promotes plasma-cell differentiation and the two proteins exist in a state of equilibrium. In the setting of BCL6 inhibition, BLIMP1 de-repression stimulates plasma cell development through a coordinated process of increasing CD38 expression and down-regulating MYC and a number of B-cell markers (17).

With respect to its essential role in GC reactions, the BCL6 expression is tightly regulated on several levels and its expression is restricted to the GC B-cells. Its regulatory mechanisms include signaling through the B-cell receptor, stimulation of the CD40 receptor by CD40 ligands expressed on T-cells, ATM-promoted BCL6 phosphorylation followed by degradation in response to massive DNA damage, acetylation and through an autoregulatory

circuit by binding to its own promoter (16). A failure of any of these negative regulatory mechanisms may lead to dysregulation or constitutive expression of BCL6 protein.

The machinery responsible for generating the somatic hypermutation of the IGHV locus may also target additional non-IGHV loci in GC B-cells such as the 5' regulatory sequences of the BCL6, c-MYC, PAX-5, PIM-1 and RhoH genes through a process known as aberrant somatic hypermutation (ASHM). ASHM has been regarded as a distinctive molecular feature of PMBL (18) and DLBCL (19). The exact role of these mutations in the pathogenesis of NHL is still unknown, but the mutational mechanism is similar to the physiological SHM occurring in the immunoglobulin variable (IGV) loci. The mutation frequency in a non-IGV locus is however, 50 to 100 times lower that of an IGV-locus (20). ASHM activity starts about 150 nucleotides downstream of the transcription start site (TSS) and typically extends upstream about two kilo bases into the gene. The 5’ non-coding region of the BCL6 gene is a frequent target of ASHM in several B-NHL entities including PMBL and DLBCL and has been implicated in their pathogenesis by disrupting the autoregularory circuit of the BCL6 gene by impairing the binding to its own promoter.

2. OBJECTIVES

1. Determine the cellular origin of primary mediastinal B-cell lymphoma based on the mutational profile of the IGHV and BCL6 genes.

The histogenetic derivation and cellular origin of PMBL has been a matter of debate for many years in the past. Once, it was considered as a subtype of diffuse large B-cell lymphoma (DLBCL), however due to its distinct clinical, pathological, and genetic features, it is now recognized as a discrete entity (21). Early immunophenotypic analysis suggested that these lymphomas originate from post germinal center B-cells (22). In later years, a GC derivation was proposed based on the BCL6 and CD10 expression, despite the fact that only a proportion of the cases express these antigens (23). The characteristic mediastinal localization and immunophenotypic similarities with thymic B-cells, led to the hypothesis of thymic origin (24, 25). Expressional profile analysis later provided evidence for a close relationship between PMBL and nodular sclerosing Hodgkin lymphoma further supporting its distinction from other subtypes of DLBCL (26, 27).

Differentiation of naïve B-cells to germinal center B-cells to plasma cells can be monitored by expression of various B-cell differentiation antigens and also by mutational analysis of IGHV gene. In order to find molecular evidence for thymic B-cell derivation of PMBL we have analyzed and compared the mutational status of IGHV gene along with the 5' noncoding region of BCL6 genes in PMBL tumor cells and thymic B-cells derived from 6 patient samples and a healthy thymus.

2. Compare the mutational landscape of the IGHV gene in lymph node and bone marrow involvement of follicular lymphoma.

Follicular lymphoma is a GC derived B-cell non-Hodgkin lymphoma characterized by the t(14;18) translocation in the majority of the cases, and by ongoing somatic hypermutation of the IGHV gene. The active hypermutational machinery results in intraclonal divergence and genetic heterogeneity of the neoplastic clones. Along with the natural progression of the

disease, neoplastic clones may invade multiple extranodal sites, including the bone marrow, where the invasion is typically characterized by paratrabecular infiltrates. The ongoing nature of IGHV gene mutations in the neoplastic clone is known to be retained during adjacent and distant lymph node propagation of the tumor (28-30), however the details of the IGHV gene mutational status in bone marrow infiltrates of FL remained unknown.

To provide insight into the BM involvement of FL we have compared the IGHV gene mutational status of paired lymph node and bone marrow samples of 3 selected cases with bone marrow involvement from 21 untreated FL patients. Simultaneous analysis of the mutational status of paired samples allowed us to reveal the clonal evolution of bone marrow involvement.

3. Correlate the mutation pattern of IGVH gene to the various grades and progression of follicular lymphoma.

Follicular lymphoma is considered as an indolent B-NHL entity with varying cytological appearance and clinical behavior. Morphologically, FLs are composed of a mixture of centrocytes and centroblasts which grow in a highly organized nodular/follicular pattern (21). Based on the proportion of centroblasts within the neoplastic follicles, FL is classified into 3 grade (grade 1-3) categories. The histological classification or grading system of FL has been shown to correlate with the clinical prognosis suggesting that FL consist of lymphomas with different biological behavior (31-33).

The ongoing nature of somatic hypermutations of IGHV genes in low grade follicular lymphomas is well established in the literature (21, 29). On the other hand, our previous studies have demonstrated the lack of IGHV intraclonal heterogenity in the transformed DLBCL cases (34, 35). It is not clear however, that at what timepoint during the clonal evolution of follicular lymphoma does this transition occur. In an attempt to correlate the IGHV molecular signature with the cytological grades of FL, we have analyzed the IGHV genes in 4 cases representing all cytological grades of FL. We evaluated the extent of intraclonal sequence heterogeneity with relation to increasing grade and progression of FL.

4. Elucidate the role of the BCL6 gene alterations in the histological transformation and clonal progression of FL.

Clonal evolution and histological transformation of FL is frequently associated with accumulation of secondary genetic alterations. The common targets include the BCL6 gene, that can be altered by chromosomal translocations and mutations clustering in its 5' noncoding region (36). These mutations can be demonstrated in a significant fraction of FL and diffuse large B-cell lymphoma (DLBCL) (37). Both the translocation and 5' noncoding region mutations disturb the orderly regulation and expression of the BCL6 gene contributing to the derailment of the physiological process of B-cell differentiation. To characterize the nature of BCL6 gene mutations in FL and to gain insight into the role of these mutations in lymphoma progression, we have analyzed the BCL6 gene in sequential biopsy specimen of 12 FL cases that either showed no sign of histological progression or that underwent morphological transformation to DLBCL.

3. RESULTS

1. Cellular origin of primary mediastinal B-cell lymphoma based on the mutational profile of the IGHV and BCL6 genes.

We have analyzed the rearrangement and somatic mutational pattern of the IGHV gene in ten independent cloned bacterial isolates of 6 primary mediastinal B-cell lymphoma patient samples and reactive thymic B-cells. The sequences obtained were compared to the corresponding reference germline sequences publicly available in the EMBL/GenBank database. Our results are summarized in **Table 1**. and **Figure 5.**

Table 1. IGHV gene rearrangement in thymic B-cells and neoplastic cells of PMBL. The statistically calculated number of R mutations in CDRs and FRs are shown in parenthesis behind the actual number of mutations.

			Ig V_H Gene						
				CDR1 and CDR2			FR1, FR2 and FR3		
Case/Clone	Frame	Closest germline gene	Nucleotide identity %	R	S	p	R	S	p
Thymic B-cells									
Clone 1	in	V1–2	94.56	3(2.94)	3	0.248	5 (9.29)	5	0.020*
Clone 2	in	V1–69	94.89	6(2.62)	0	0.025*	6 (8.72)	3	0.076
Clone 3	in	V3–33	92.85	7(4.04)	2	0.057	8 (11.9)	4	0.038*
Clone 4	in	V3–30	96.59	1(1.92)	1	0.280	5 (5.58)	3	0.229
Clone 5	out	V3–23	90.08	10(4.8)	1	0.007*	13 (15.6)	3	0.090
Clone 6	in	V3–8	93.54	15(5.6)	0	0.001*	3 (9.23)	1	0.002*
Clone 7	in	V3–48	97.95	4(1.08)	1	0.010*	0 (3.46)	1	~
Clone 8	in	V5–51	90.08	4(4.88)	4	0.191	13(15.9)	6	0.079
Clone 9	in	V5–51	92.18	6(4.03)	4	0.110	8 (13.6)	5	0.010*
Clone 10	in	V5–51	93.54	5(3.34)	2	0.131	11(11.2)	1	0.181
PMBL									
Case 1	out	V3–30	100	0(0.00)	0	0.000	0(0.00)	0	0.000
Case 2	in	V5–51	95.8	4(1.93)	1	0.081	5(6.51)	1	0.155
Case 3	in	V3–20	86.1	15(6.45)	5	0.001*	8(21.2)	9	0.001*
Case 4	in	V4–34	87.15	20(6.86)	0	0.001*	14(21.2)	3	0.008*
Case 5	in	V3–7	89.23	4(5.58)	2	0.156	15(18.7)	11	0.059

*With statistical significance ($P < 0.05$)

Table 1. displays the degree of similarity of the cloned sequences to the closest germline genes found in the database. As anticipated, the reactive thymic B-cells represent

polyclonal populations with differing heavy chain rearrangement indicated by multiple IGHV usage in the selected clones. In the thymic B-cells, all but one V_H-D-J_H sequences were in-frame, without the presence newly generated stop codons, suggesting that 9 out of the 10 clones was able to express functional B-cell receptor. The VH sequences of these cells showed 90.08% to 97.95% homology to their closest germline compatible with mutated IGHV status and previous exposure to the somatic mutational machinery of the GC.

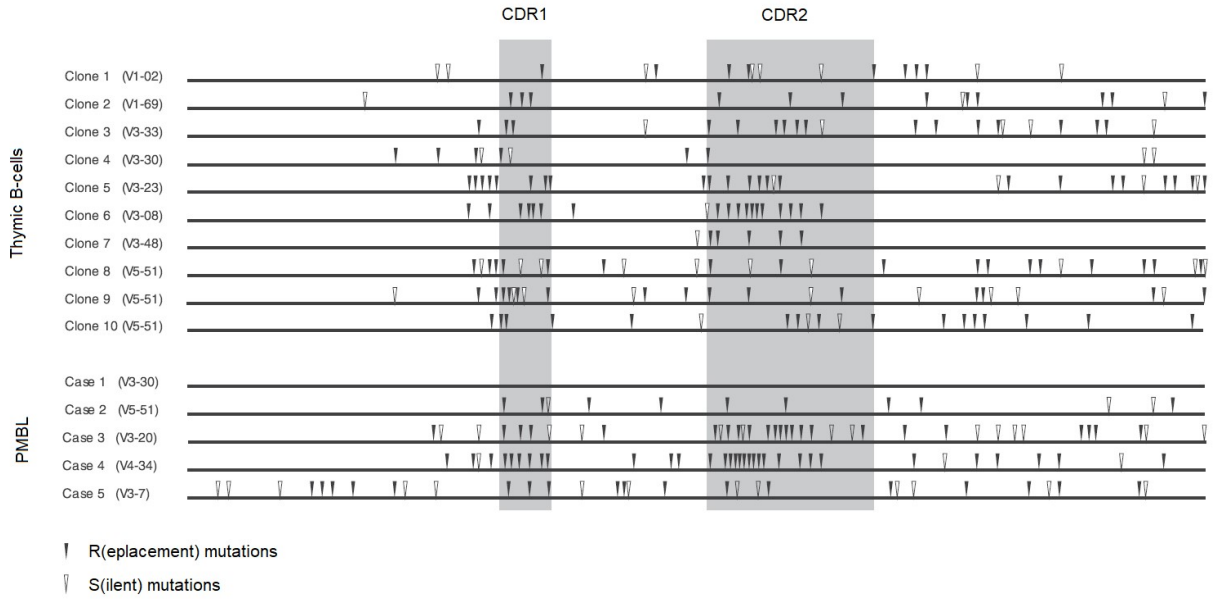

Figure 5. Distribution of mutations in VH gene sequences rearranged by thymic B-cells and tumor cells of MBLs.

The PCR analysis was able to amplify V_H-D-J_H rearrangements in only five out of the six PMBL cases. In all 5 cases, only identical and unique sequences were obtained from multiple cloned bacterial isolates proposing a monoclonal IGHV rearrangement. Four of the VH sequences were in frame and one was out of frame. The case displaying a frame shift as a consequence of nucleotide deletion or insertion could not express a functional BCR. Case 6 possibly had a mutation in either the FR1 or FR4 regions of the VH gene involving the primer binding site preventing proper annealing of the PCR primers. Interestingly one of the cases, utilizing a V_H3 family germline gene, showed 100% sequence homology to the

corresponding germline. The other four cases displayed 86.1%-95.8% sequence homology with the germline, compatible with mutated IGHV status.

Table 1. summarizes and **Figure 5**. also shows the number and distribution of observed and statistically calculated silent and replacement somatic mutations of the CDR and FR regions of IGVH gene. The statistical calculation was performed according to the binomial distribution model described by Chang and Casali (38). This method allows the determination of the presence or absence of antigenic selective pressure in the generation of somatic mutations. The formula basically calculates the predicted number of replacement mutations in the CDR and FR regions of the IGHV gene based on the total number of mutations, length and distribution of the CDR and FR sequences. These calculated values are shown in parenthesis in **Table 1**. behind the actual number of mutations found. Antigenic selection alters the pattern of acquired random somatic mutations during the germinal center transit of B-cells by selectively increasing the number of replacement IGHV mutations of the CDRs, while keeping the number of these mutations low in the conserved FR regions. Thus, we can collect genetic evidence for antigen selection and transit through a GC-like environment by statistically comparing the number of actual and calculated mutations of the CDR and FR regions.

The binomial distribution model revealed molecular evidence for clonal selection (significantly more than expected replacement mutations in the CDR regions) in four sequences of the thymic B-cells (clones 2, 5-7) and in two PMBL cases (case 3 and case 5). With respect to the FR regions, the analysis revealed fewer than expected mutations in five thymic B-cell clones (clones 1,3,6,7 and 9) and two PMBL cases (case 3 and case 5) suggesting significant conservation of these sequences.

To determine the presence of aberrant somatic hypermutations the 5' noncoding regulatory regions of the BCL6 gene were cloned and sequenced in both the thymic B-cells and the six PMBL cases. The 998 base pair long intron upstream from the first noncoding exon was amplified using 5 separate, overlapping PCR reactions (E1.9-E1.13) and subsequently cloned. Ten independent bacterial colonies from each reaction were sequenced and compared to the respective germline sequences available from EMBL/GeneBank

database. The BCL6mutations found are depicted in **Figure 6**. The reactive thymic B-cells harbored a total of 22, while the six PMBL cases contained 18 mutations. An overall mutation frequency of 3×10^{-3}/bp was calculated for the PMBL cases, a value that compares favorably with previous reports (23). The detected mutations included single base pair substitutions and insertions. We observed 2 polymorphisms in the thymic B-cells. The polymorphism at position 118 (G>C) was shared by all thymic B-cells, while the insertion at position 860 (insA) was only present in 3 cases. We also found a polymorphism of the germline sequence at position 976 (C>G) in four of the PMBL cases. The distribution of the somatic mutations and gene polymorphisms affecting the 5' noncoding region of the BCL6 gene is depicted in **Figure 6.**

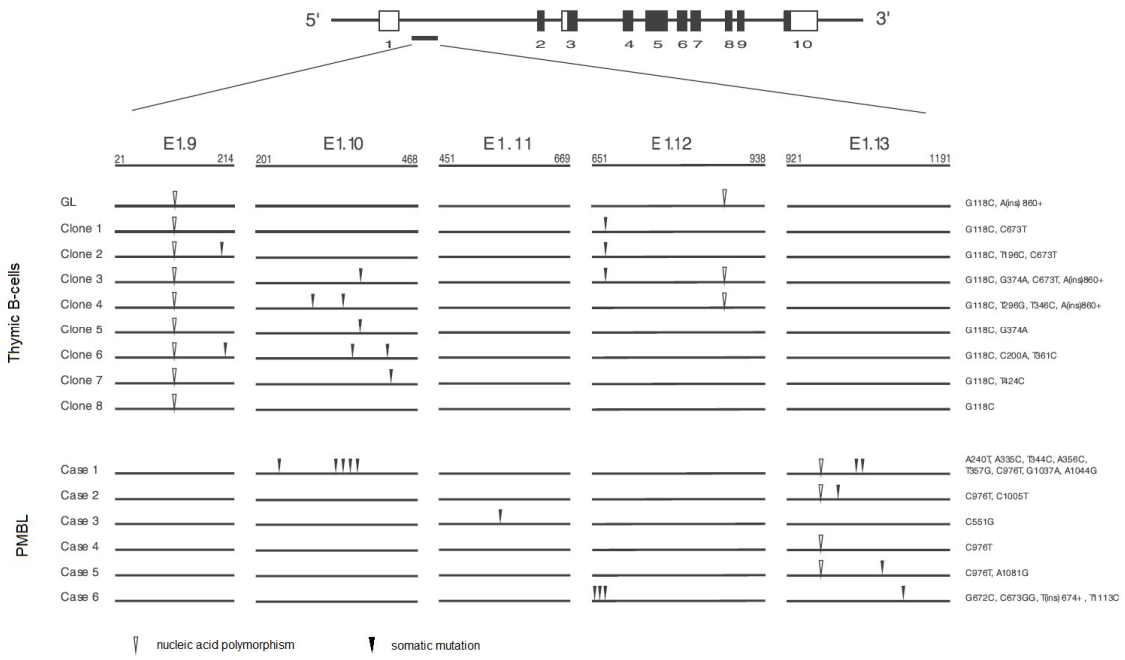

Figure 6. Schematic representation of the BCL-6 gene and distribution of mutations within the BCL6 5' noncoding sequences of thymic B-cells and tumor cells of PMBL.

2. Comparison of the IGHV mutational profile of lymph node and bone marrow involvement of follicular lymphoma.

To gain insight into the characteristics of bone marrow involvement of follicular lymphoma, we compared the cytological grades, growth pattern and immunophenotype of the tumor cells from paired lymph node and bone marrow samples of 21 untreated patients with FL. We also performed mutational analysis of the IGHV genes of the LN and respective BM specimens in three selected cases to evaluate and compare the mutational status of the IGHV gene in these different anatomical locations and to reveal the paths of clonal evolution.

The detailed analysis of the cytological grades, immunophenotype, growth pattern of the bone marrow infiltrate and the various possible paths of clonal evolution of FL is described in the original publication (39). Here we would like to focus our attention on the somatic mutation patterns of the IGHV genes in the lymph node and bone marrow involvement of follicular lymphoma.

The PCR amplification of the IGHV genes showed that the V_H-D-J_H rearrangements were identical in all clones of the LN and BM pairs providing molecular evidence for common clonal origin of the tumor cells in lymph node and respective bone marrow samples. Following the TA cloning of the PCR products, sequence analysis was performed to determine the closest germline IGHV genes using the IMGT/V-QUEST and the NCBI/BLAST databases.

As expected, the sequences obtained from each lymph node specimen differed from each other by a various number of distinct mutations compatible with the ongoing nature of somatic hypermutations in this germinal center derived lymphoma entity. The number of detected IGVH mutations ranged from 23-42 in the 53 lymph node clones investigated. A significant proportion of these mutations were shared between all clones, while the number of unique mutations ranged from 0-7. This data indicates that several of the lymph node clones contained only shared mutations. Interestingly the genetically identical subclones with only shared mutations were identified only in grade 3 cases, possibly suggesting the

decreased intraclonal heterogeneity. The binomial distribution model revealed fewer R mutations in the FRs and more R mutations in the CDRs than could be expected due to chance in all investigated LN clones (data shown in original publication) indicating presence of antigen selection.

Table 2. Summary of mutational analysis of paired lymph node and bone marrow samples from 3 selected follicular lymphoma cases.

	Closest germline IHGV gene	LYMPH NODE				BONE MARROW			
		Number of IGHV mutations			Cytological grade	Number of IGHV mutations			Cytological grade
		All	Shared	Unique		All	Shared	Unique	
Case 1	VH3-53	37-42	33	4-7	G2	36-42	33	3-9	G2
Case 2	VH3-21	23-30	23	0-7	G3	21-27	18	3-9	G2
Case 3	VH3-7	23-30	23	0-7	G3	26-39	2	24-37	G1

The samples originating from the bone marrow biopsies also showed intraclonal sequence diversity in all three investigated BM cases. The number of detected IGVH mutations ranged from 21-42 in the 57 bone marrow clones investigated. The sequence deviations from the germline were results of single nucleotide changes in all cases, and despite these mutations potentially functional genes were produced. The Chang and Casali binomial distribution analysis (38) revealed genetic signature of antigen selection as the number of detected mutations in the CDR regions of the bone marrow clones form all three cases exceeded the calculated values (data shown in original publication). The closest germline genes, the range of detected IGHV mutations together with the number of shared and unique mutations found in the lymph node and bone marrow clones are shown in **Table 2.**

Evaluation and comparison of the somatic mutations between the lymph node and bone marrow samples of the same patient revealed the presence of shared and unique mutations of the IGHV gene. Some of these unique bone marrow mutations were found in

multiple subclones (subclonal heterogeneity) of the bone marrow infiltrate, while they were absent in the lymph node clones. These data suggest the presence of ongoing somatic mutation in a location independent from the lymph node GC reaction.

3. Mutational pattern of IGVH gene in the different grades of follicular lymphoma

Using individual Ig V_H gene family-specific leader sense primers in conjunction with the consensus J_H antisense primer, the Ig V_H-D-J_H segments were PCR-amplified from cDNA in six independent reactions in 4 cases representing different grades of FL. The PCR products were cloned, and twelve independent bacterial isolates (labelled A-L) were sequenced and analyzed from each case. The nucleotide and deduced amino acid sequences of the V_H-D-J_H gene segments, and those of the closest respective germline gene sequences are summarized in **Table 3.**

Table 3. Analysis of Ig V_H-D-J_H Genes Expressed by Neoplastic Cells of Grade 1- Grade 3 FL Cases.

			Ig V_H Gene									
Case	Clone	Intraclonal Diversity	Closest Germline Gene	Nucleotide Identity %	CDR1 and CDR2			FR1, FR2 and FR3			D Gene	J_H Gene
					R	*p*	S	R	*p*	S		
G1 FL	A-C	yes	IGHV5-51	100.0	0 (0.00)	0.00	0	0 (0.00)	0.00	0	ND	HJ4
	D-L	yes	IGHV5-51	97.6	5 (1.74)	$1.56x10^{-2}$*	3	2 (5.94)	$1.17x10^{-2}$*	0	ND	HJ4
G2 FL	A-C	yes	IGHV3-48	93.9	9 (3.26)	$1.68x10^{-3}$*	3	4 (10.28)	$2.33x10^{-3}$*	2	ND	HJ4
	D	yes	IGHV3-48	93.6	10 (3.44)	$5.78x10^{-4}$*	3	4 (10.85)	$1.27x10^{-3}$*	2	ND	HJ4
	E	yes	IGHV3-48	93.9	9 (3.26)	$1.68x10^{-3}$*	3	4 (10.28)	$2.33x10^{-3}$*	2	ND	HJ4
	F	yes	IGHV3-48	93.2	10 (3.62)	$9.47x10^{-4}$*	3	4 (11.42)	$6.79x10^{-4}$*	3	ND	HJ4
	G	yes	IGHV3-48	92.9	14 (3.80)	$1.00x10^{-6}$*	3	2 (11.99)	$7.00x10^{-6}$*	2	ND	HJ4
	H	yes	IGHV3-48	92.6	15 (3.98)	$1.00x10^{-6}$*	3	2 (12.56)	$3.00x10^{-6}$*	2	ND	HJ4
	I	yes	IGHV3-48	92.2	15 (4.16)	$1.00x10^{-6}$*	3	2 (13.13)	$2.00x10^{-6}$*	3	ND	HJ4
	J	yes	IGHV3-48	92.6	14 (3.98)	$3.00x10^{-6}$*	3	3 (12.56)	$3.00x10^{-5}$*	2	ND	HJ4
	K	yes	IGHV3-48	91.8	14 (4.34)	$1.10x10^{-5}$*	5	3 (13.70)	$7.00x10^{-6}$*	2	ND	HJ4
	L	yes	IGHV3-48	91.1	15 (4.71)	$6.00x10^{-6}$*	4	5 (14.84)	$7.70x10^{-5}$*	2	ND	HJ4
G3 FL	A-L	no	IGHV3-30	91.8	8 (4.28)	$3.25x10^{-2}$*	4	9 (13.78)	$2.43x10^{-2}$*	3	ND	HJ4
G3 FL	A-L	no	IGHV4-39	90.2	7 (5.22)	$1.12x10^{-1}$	3	14 (15.98)	$1.13x10^{-1}$	4	ND	HJ6

Abbreviations: G1 FL, grade 1 Follicular Lymphoma; G2 FL, grade 2 Follicular Lymphoma; G3 FL, grade 3 Follicular Lymphoma; ND, not detected; R, number of detected and (expected) replacement mutations; S, number of detected silent mutations; *p*, probability; *, statistically significant (P<0,05).

In the grade 1 FL case (G1), V_H-D-J_H gene sequences revealed two unique, but collinear sequences (G1/A-C and G1/D-L) sharing the same V_H, (D), and J_H genes and joinings. These IGVH sequences displayed the highest degree of identity to IGHV5-51 and HJ4 germline genes. The intervening sequence could not be attributed to any germline D gene. The expressed IGVH genes of clones A-C were 100% identical to those of the germline IGHV5-51 gene, but clones D-L displayed 10 nucleotide differences when compared to germline (97,6% sequence homology).

The grade 2 FL case (G2) showed 10 unique, but collinear V_H-D-J_H sequences sharing the same V_H, (D), and J_H genes and joinings. These IGHV sequences displayed closest correlation with the germline IGHV3-48 and HJ4 genes. The intervening sequences could not be attributed to any germline D gene. The expressed IGHV genes revealed 91.1-93.9% homology to the reported germline sequence.

In the first grade 3 FL case (G3) the IGHV sequences in all bacterial isolates were found to be identical with each other, and showed a 24 nucleotide difference to the germline (91.8%. sequence homology). The highest degree of identity was with the IGHV3-30 and HJ4 germline genes. The intervening sequences could not be attributed to any germline D gene.

In the other G3 FL case the IGHV gene sequences derived from the tumor cells were also identical. The expressed genes displayed the highest degree of identity to those of the germline IGHV4-39 and HJ6 genes. The intervening sequence could not be attributed to any germline D gene. The IGHV gene sequence showed a 28 nucleotide difference from the reported germline, that translated into a 90,2% sequence homology with the germline.

In all the four FL cases, the expressed IGHV sequences were aligned and the sequence of their putative FL cell progenitor was calculated. From the pattern of shared and unique mutations - assuming that shared mutations represent single events and not independent mutations - genealogical trees of the evolution of FL cells were constructed, and putative progenitor IgH gene sequences were deduced **(Figure 7.)**.

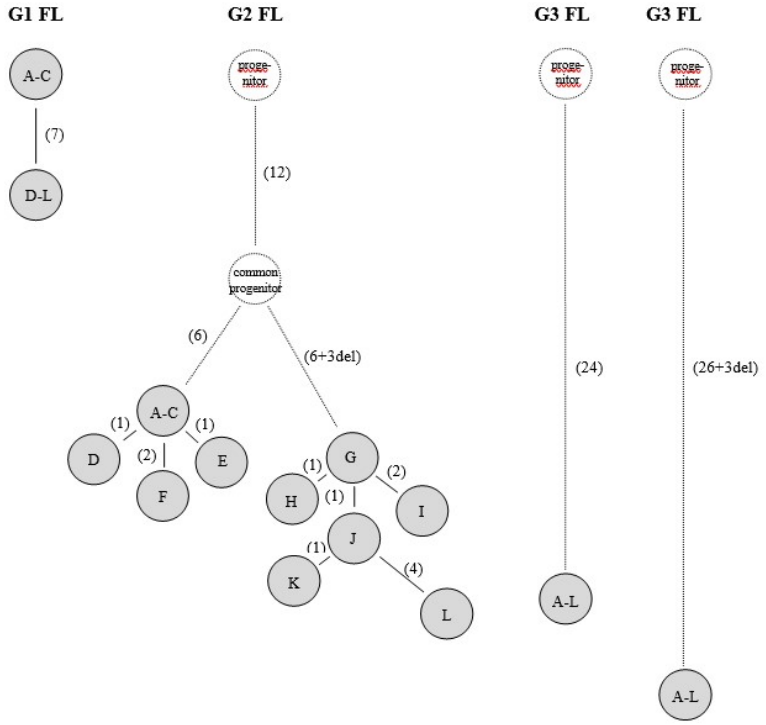

Figure 7. **Clonal evolution of four FL cases.** Genealogical trees are constructed from the pattern of shared and unique mutations of Ig V_H-D-J_H genes, assuming that shared mutations represent single events. The assumed common progenitors and the intermediates are drawn as dotted circles. The number of mutations separating each branch are given. The distances between the circles are proportional to the number of mutations.

To rule out the confounding effects of single nucleotide polymorphisms, and to verify that the mutations were acquired following IgH rearrangement genomic DNAs from the tumor samples of all four patients were PCR-amplified using sense primers specific for germline FR1 sequences in conjunction with antisense primers specific for V_H gene family heptamer recombination signal sequences. Thus, our PCR reactions were specifically designed to only amplify the germline configurations of the V_H genes, since the heptamer recombination signal sequences are lost in the rearranged IgV_H genes. Using these oligonucleotide primer pairs, we were able to obtain PCR products of the appropriate size in

all four cases, indicating that the tumor samples contained some non-neoplastic cells with germline IGHV configuration. In each case, the PCR products were cloned, and plasmid DNAs originated from six independent bacterial isolates were sequenced. The nucleotide sequences were 100% identical to the respective germline genes from the EMBL/GeneBank database ruling out the confounding effects of SNPs.

To determine whether remaining unmutated FL progenitors are still present among the somatically mutated FL cells, or these cells were completely lost during the clonal evolution of FL, we developed clonotype specific PCR reactions specific for the unmutated and expressed IgV_H gene sequences. For this PCR amplification we used the germline CDR1 sequence specific sense primers in conjunction with the consensus J_H antisense primer. No DNA amplifications were detected using this approach suggesting that the unmutated common progenitor cells were lost during the clonal evolution in all our FL cases.

The number of expected replacement (R) mutations and the probability that these R mutations in the CDR or FR regions arose by chance was calculated for all four FL cases **(Table 4.).** In G1, G2 and one of the G3 cases the number of R mutations in the CDR1 and CDR2 regions were significantly higher than expected ($p<0,05$), while in the FR1, FR2 and FR3 regions they were lower than expected. This indicates that the likelihood that these R mutations in the CDRs and FRs occurred randomly is low. Thus, the results are consistent with antigen selection in clones of G1/D-L, G2/A-L and G3, providing genetic evidence that these IGHV gene segments were under positive selective pressure to mutate their CDRs, but negative pressure to mutate their FRs. In the remaining G3 case the number of R mutations in the CDR and FR were similar to that expected by chance alone, indicating the lack positive selective pressure of antigen selection.

4. The role of the BCL6 gene somatic mutations in the histological transformation and clonal progression of follicular lymphoma

Clonal evolution and histological transformation of FL is frequently associated with the accumulation of secondary genetic alterations. It has previously been demonstrated that the BCL6 gene can be altered by chromosomal rearrangements and by mutations clustering in its 5' noncoding region in a significant proportion of FL and diffuse large cell lymphoma (DLBCL) (36, 37, 40, 41). Approximately 70% of DLCL, 45% of FL, 58% of AIDS related NHL, 39% of Burkitt's lymphoma, and 44% of posttransplant lymphoproliferative disorders carry BCL6 genes mutated in the 5' noncoding region (42, 43). These mutations are of somatic origin and frequently biallelic.

To elucidate the role of the BCL6 gene alterations in the histological transformation and clonal progression of FL, we analyzed serial biopsy specimens from 12 patients with FL. In two of the analyzed cases of FL showed no histological transformation in the second biopsy, and 10 cases showed transformation to DLBCL in the second biopsy.

No BCL6 gene translocation was detected in any of our selected samples. We found a total of 58 mutations in the 5' noncoding region of the BCL6 gene in seven cases. In five cases, the mutations were present in both the original FL and the clonally related FL or DLBCL biopsies. In two cases only the DLBCL samples contained mutations, while the BCL6 gene was unmutated in the original FL. The BCL6 mutations were identical in the first and second biopsy specimens of the 2 FL cases that did not show morphological transformation. We found considerable intraclonal sequence heterogeneity in six patients where FL underwent morphological transformation, indicating an ongoing type of somatic mutation. We performed PCR-SSCP analysis to screen for the presence or absence of mutations in four separate PCR reactions (E1.10-E1.13). The mutated sequences were identified by altered electrophoretic migration pattern in the polyacrylamide gel. The results are shown in **Table 4.**

Table 4. Summary of the BCL6 gene mutations. PCR-SSCP analysis of the BCL6 5' noncoding regions in 12 cases of paired FL and subsequent FL or DLBCL samples.

Case	Sample	Histology	BCL6 5' non-coding region				BCL6 expression (%)
			E1.10	E1.11	E1.12	E1.13	
1	A	FL-II	WT	M	M	WT	100
	B	DLBCL	WT	M	WT	WT	100
2	A	FL-II	WT	M	WT	WT	100
	B	DLBCL	WT	M	WT	WT	100
3	A	FL-III	WT	M	WT	M	100
	B	DLBCL	WT	M	WT	M	100
4	A	FL-III	WT	WT	WT	WT	100
	B	DLBCL	WT	WT	WT	WT	100
5	A	FL-I	WT	M	WT	WT	100
	B	DLBCL	WT	M	WT	WT	20
6	A	FL-I	WT	WT	WT	WT	100
	B	DLBCL	WT	M	M	WT	10
7	A	FL-I	WT	WT	WT	WT	100
	B	DLBCL	M	M	WT	WT	10
8	A	FL-I	WT	WT	WT	WT	100
	B	DLBCL	WT	WT	WT	WT	100
9	A	FL-II	WT	WT	WT	WT	100
	B	DLBCL	WT	WT	WT	WT	100
10	A	FL-I	WT	WT	WT	WT	ND
	B	DLBCL	WT	WT	WT	WT	ND
11	A	FL-I	WT	M	WT	WT	100
	B	FL-I	WT	M	WT	WT	100
12	A	FL-II	WT	WT	WT	WT	100
	B	FL-II	WT	WT	WT	WT	100

A: first biopsy sample; B: second biopsy sample; FL-I: follicular lymphoma—grade 1; FL-II: follicular lymphoma—grade 2; FL-III: follicular lymphoma—grade 3; DLBCL, diffuse large B-cell lymphoma; M: mutations identified by PCR-SSCP fragments; WT: wild-type; ND: not detected.

Expression of the BCL6 protein was detected by immunohistochemistry using BCL6 monoclonal antibodies. The staining pattern was nuclear. The BCL6 expression of the tumor samples is summarized in **Table 4**. In all FL and in six of the DLBCL samples, the 100% of the tumor cells expressed the BCL6 protein. BCL6 expression was reduced to 10 to 20% in three transformed DLBCL samples (Cases 5–7).

To confirm the presence of mutations affecting the BCL6 5' noncoding region, we cloned and sequenced the pairs of PCR products that showed altered migration pattern with PCR-SSCP analysis (Cases 1-3, 5-7 and 11). Six independent bacterial isolates were analyzed for each sample and compared with germline sequences of the BCL6 5' noncoding region found in the EMBL/GeneBank database. The sequencing results are shown in **Table 5.**

	Case 1		Case 2		Case 3		Case 5		Case 6		Case 7		Case 11	
Fragment	A	B	A	B	A	B	A	B	A	B	A	B	A	B
E1.10	–	–	–	–	–	–	–	–	–	–	–	242 (C → G)	–	–
											–	285 (A → G)		
											–	259 (T → C)		
											–	285 (T → A)		
E1.11	–	523 (G → A)	531 (G → C)	–	504 (T → C)*	–	551 (G → C)*	551 (G → C)	–	586 (C → G)	–	552 (G → A)*	481 (A → C)	481 (A → C)
	–	554 (G → T)	–	551 (G → C)	–	522 (A → G)*	613 (C → G)*	–	–	588 (G → A)	–	577 (C → G)*		
	586 (C → G)*	–			–	533 (T → G)*	614 (T → A)*	–	–	602–633 (del)	–	604 (T → C)*		
	602 (G → A)*	–			–	544 (A → C)*	624 (T → C)*	–			–	638 (T → C)*		
					–	553 (A → C)*	635 (T → C)*	–						
					–	577 (C → G)*								
					–	580 (T → C)*								
					–	581 (T → C)*								
					583 (C → G)*	–								
					–	585 (G → A)*								
					–	588 (G → A)*								
					–	590 (T → C)*								
					593 (T → G)*	–								
					–	596 (C → T)*								
					–	599 (A → G)*								
					–	604 (T → G)*								
					–	634 (T → G)*								
					636 (T → C)*	–								
	–	–	–	–	–	–	–	481 (A → T)	–	626 (C → A)*	–	556 (G → C)		
							–	482 (G → A)	–	637 (T → C)*				
E1.12	839 (T → C)	–	–	–	–	–	–	–	–	772 (G → T)	–	–	–	–
E1.13	–	–	–	–	–	946 (T → C)*	–	–	–	–	–	–	–	–
					–	973 (T → C)*								
					976 (C → T)*	976 (C → T)*								
					1054 (T → C)*	–								
					–	1109 (C → G)*								
					–	1116 (C → T)*								
					1137 (G → C)*	–								

A, first biopsy sample; B, second biopsy sample.
*Mutation is associated with intraclonal divergence.
–, not mutated.

Table 5. Distribution of mutations within the BCL6 5' noncoding region in seven cases of paired FL and subsequent FL or DLBCL samples.

4. DISCUSSION

1. Reactive thymic B-cells contain mutated IGHV and BCL6 genes compatible with follicular/postfollicular origin possibly through a unique developmental process.

Thymic B-cells represent a unique population of B lymphocytes located at the cortico-medullary junction of the thymus, a highly specialized organ for T-cell development. Thymic B-cells are distinct from peripheral B-cells both in terms of their origin and phenotype. Several lines of evidence suggest that they develop within the thymus from B-lineage committed progenitors and are not recirculating peripheral B-cells. (44). The existence of this subset of B-cells was first described in 1987 by Isaacson (45). Based on their localization and phenotype, thymic B-cells are thought to play a physiological role as APCs for establishing central T-cell tolerance (46-48). Recent evidence also suggests that thymic B-cells might be involved in regulatory T cell induction (49).

The cellular origin of thymic B-cells is still a matter of debate. Thymic B-cells can be detected very early in ontogeny, even at day 18 of embryonic life, prior to the onset of B-cell development in the bone marrow (45, 50), suggesting that these B-cells may develop independently from the bone marrow. Identification of a B-cell progenitor-like population within the thymus also gave support to the idea that there may be a distinct intrathymic pathway for B-cell development (51). Most of our knowledge about thymic B-cell development comes from the analysis of fairly differentiated stages of B-lineage development. It is difficult to make definitive statements about the lineage of these cells as we trace this developmental pathway back towards its earlier progenitors (44).

To gain insight into the cellular origin of human thymic B-cells, we analyzed the IGHV and BCL6 5' noncoding region sequences of reactive thymic B-cells. All ten cloned IGHV sequences originating from polyclonal thymic B-cells carried a high load of somatic mutation with the mean base exchange rate of 6.41%. This value is considerably higher than the 3–4% average IGHV mutation frequency rate of non-malignant GC and memory B-cells (52). The binomial distribution analysis indicated that in 7/10 clones analyzed, the distributions of R mutations in the CDRs and/or FRs were compatible with antigen selection

or, in other words, the thymic B-cells have undergone affinity maturation. In the remaining three cases the distribution of R mutations in both CDRs and FRs were random, consistent with mutated, but antigen naïve B-cells. These findings support the idea that B-cells of the adult thymus contain mutated IGHV genes and some of these B-cells show evidence for affinity maturation. Both of these IGHV mutational patterns are compatible with postfollicular origin of thymic B-cells.

The nucleic acid sequence analysis of 5' noncoding region of the BCL6 gene in thymic B-cells also revealed presence of frequent mutations, further supporting the similarities between thymic B-cells and GC/post-GC B-cells. There are 2 possible explanations for the existence of both IGHV and BCL6 mutations in thymic B-cells. One explanation suggests that these somatic mutations are generated in GC of the lymph nodes and the mutated B-cells later migrate to the thymus. This hypothesis would be supported by the fact that the normal thymus does not contain histologically identifiable lymphoid follicles. Alternatively, it is also possible that the process of somatic hypermutation in B-cells is not limited to the GC of lymph nodes and the thymus provides an appropriate structural and functional microenvironment where B-cells can undergo the hypermutation process. Several studies provide support for this theory. Bodely et al described the existence of dendritic type accessory cells in the mammalian thymus supporting a favorable microenvironment for antigen presentation (53). The findings that peripheral B-cells inefficiently migrate to the thymus when splenocytes are injected intravenously further supports the idea that B-lymphocytes do not readily recirculate to the thymus from the periphery (48, 54). The idea of an alternative GC-like microenvironment in the thymus is also supported by the findings of Flores et al. (55), who found clonally related diverging VH-D-JH sequences among B-cell population in the thymus. The analysis of BCL6 gene mutations in our study also support this hypothesis, since at certain nucleic acid positions three clones shared identical mutations, which were absent in the germline sequences suggesting locally generated intraclonal heterogeneity. Thus our results indicate that reactive thymic B-cells show mutated, follicular/postfollicular type IGHV and BCL6 gene sequences, and that the development of thymic B-cells is unique in a way that the somatic mutations

may, at least be partially generated through independent mechanisms from the lymph node germinal centers.

2. The somatic mutation pattern of IGHV and BCL6 genes support postfollicular origin of primary mediastinal B-cell lymphoma.

The analysis of IgH gene in PMBL patient samples revealed amplifiable VH-D-JH rearrangements in five of the six cases. We failed to amplify the IGHV gene segments in one case, which may be consequence of unsuccessful VH-D-JH gene rearrangement or somatic mutations affecting the PCR primer binding sites. In one case, where the IGHV gene sequences showed 100% homology to the closest germline gene the VH-D-JH rearrangement was out-of-frame, possibly resulting in a non-functional rearrangement, explaining the complete lack of somatic mutations. The remaining four cases were in frame suggesting functional gene products. In these four PMBL patients the IGHV genes carried a high load of somatic mutation. This finding is in-line with results of other studies reporting higher than average somatic mutation rate for PMBL as compared to other B-NHL (56-59). The mean base exchange rate of the sequenced clones was 10.43%, which somewhat exceeds the mutational frequency found in human thymic B-cells. The high load of somatic mutations found in PMBL suggests that these cells have undergone a transfollicular or analogous differentiation and maturation process.

Somatic hypermutation of the BCL6 gene is also considered as a specific marker for B-cells to transit through the germinal center. The 5' noncoding region of BCL6 gene was mutated in five of the six PMBL cases analyzed in this study, further supporting the idea that PMBLs originate from GC/post-GC B-cells. Interestingly, high loads of mutations were found even in those cases where IGHV gene was unmutated in association with a frame-shift or in the case where PCR amplification failed to amplify the VH-D-JH sequence. Other series of studies also reported coexistence of BCL6 mutations parallel to IGHV mutations with variable proportions of PMBL. Pilleri et al. (23) and Malpeli et al (60) both describe simultaneous presence of BCL6 mutation in about 50% of the cases in larger series of PMBL.

The variability in the mutational status of the BCL6 gene demonstrated by different authors may point to diverse histogenetic pathways for PMBL. Furthermore, other B-cell NHL entities with mutated IGHV genes do not carry a mutated BCL6 genes suggesting that the mutation process affecting the IgH and BCL6 loci may not necessarily occur in tandem (61, 62). Similar heterogeneity of the mutation patterns is seen in normal circulating memory B-cells, where 80–100% of the cells carry mutations of the IGHV, while only about 40% of these cell display mutations of the BCL6 gene (61).

In summary, our findings provide molecular evidence that both the normal thymic B-cells and the tumor cells of PMBL carry mutated IGHV sequences indicating that both of these mature B-cell populations have transited through the germinal center or a similar mutation favoring microenvironment at one point in their differentiation process. The similarities of the mutation patterns of IGHV and BCL6 genes observed in the reactive thymic B-cells and PMBL tumor cells, support the hypothesis that PMBL originates from thymic B-cells.

3. Low-grade FLs demonstrate ongoing somatic hypermutation in line with follicular origin, while high-grade FL shows lack of intraclonal heterogeneity suggesting termination of ongoing hypermutation compatible with post-follicular like phenotype.

Follicular lymphoma is classified into grade 1-3 categories based on its histological features according to the revised WHO classification of hematopoetic diseases (21). The different grades of FL show significant correlation with clinical outcome (33, 63). Several large studies have identified numerous recurrent mutations affecting B-cell receptor signaling, differentiation, cell cycle regulation, derangements of epigenetic modification and immune evasion that can be linked to clinical progression of the disease (64-67), however the mechanism of clonal evolution is still a matter of debate (68, 69). The IGHV somatic hypermutation based spatial evolution of follicular lymphoma have been demonstrated previously (70). In our study we have analyzed the sequence of Ig V_H-D-J_H genes in different histological grades of FL in an attempt to reveal differing molecular signatures correlating

with the histological appearance and biological behavior. We have found that grade 1-2 FLs (low-grade FLs), and grade 3 FLs (high-grade FLs), express Ig V_H-D-J_H gene sequences with different patterns of somatic mutation. The presence of ongoing somatic mutation/intraclonal diversity was detected in the low cytological grades indicating that the tumor cells are still under the influence of the mutation machinery, possibly generated through interactions with their environment in the GC or GC analogous milieu. In contrast, grade 3 FLs expressed mutated, but homogenous V_H-D-J_H gene sequences, suggesting that the previously active mutational mechanism is already terminated, and the neoplastic cells have become less dependent on GC-like environmental stimulation for survival.

The process of active somatic hypermutation of IgH genes, which takes place in germinal centers during normal B cell differentiation, has been suggested to be a common feature of FLs (70-73). Our results only partially support these observations. According to our analysis, the low-grade FLs showed extensive ongoing somatic hypermutation of the IGHV genes with significant intraclonal heterogeneity. The V_H genes of the neoplastic clones showed 93.9-91.1% sequence homology to the germline with several clonally related, but intraclonally divergent sequences. The shared and non-shared nucleotide alterations detected in the different neoplastic subclones suggest a stepwise accumulation of mutations paralleling the evolution of the FL clones. These findings are congruent with previous reports, and confirm that the clonal expansion of low-grade FLs is associated with continuously ongoing somatic hypermutation of the IGHV gene; the characteristic mutation pattern found in B-NHL entities of follicular center origin.

The intensely mutated, but homogenous V_H-D-J_H gene sequences detected in grade 3 FLs are strikingly different from the intraclonally divergent subclones of low-grade FLs. The IGHV gene sequences expressed by grade 3 FLs revealed 24-29 nucleic acid differences (translating to 90.2%-91.8% sequence homology) when compared to the germline without molecular evidence of intraclonal heterogeneity. These findings reveal a previously agile, but currently inactive mutational machinery in high-grade FLs compatible with a stable mutated IGHV signature usually characterizing mature NHLs originating from the post-follicular stage of B-cell development.

Our study demonstrated markedly differing somatic mutational patterns of the IGHV gene sequences between low-grade and high-grade FLs. Interpretation of our findings offers two possible alternative explanations for tumor clone development that clarifies the contrast between the distinct somatic mutation patterns associated with different grades FL.

In the first model, FL originates from a single committed progenitor driven by the presence of t(14,18) translocation, and following malignant transformation, ongoing (aberrant) somatic hypermutation gives rise to sequential appearance of different grades of FLs. In this model, the various grades of FL merely represent different time points in the clonal evolution of the tumor. This hypothesis is supported by several findings. Our cases demonstrate that the diverse mutated FL subclones may originate from common unmutated progenitor clonotypes, and that according to our results, the number of mutations show progressive increase with grade. In this model, the development of grade 3 FL originates from a late neoplastic clone with numerous previously acquired IGHV gene mutations, rather than from the outgrowth of an early more germline-like clone. In this setting, more V_H gene mutations translate to longer exposure time to the highly mutagenic GC environment, which also allows the stepwise accumulation of transforming mutations of the key regulatory genes through aberrant somatic hypermutation. Lines of evidence supporting the clonal relationship between the original FL and the subsequent DLBCL arising through its high-grade transformation is readily available from the literature (68, 74, 75). In this scenario, the early phases of progression could be associated with ongoing somatic mutation that may freeze-up during the process of high-grade transformation together with loss of the environmental interactions or possibly the functional loss the mutation machinery.

In an alternative model, FL is a heterogeneous disorder originating from different committed precursor cells at various stages of B-cell development. Based on this hypothesis, grade 1 and 2 FLs are transformants of germinal center B-cells, while grade 3 FL originates from a post-follicular (memory) B-cell. This hypothesis is also supported by the finding that we were unable to confirm the presence of the common progenitor clonotype in grade 3 FL by clonotype specific PCR amplification. A recent demonstration that *in situ* follicular lymphoma (ISFL), overt FL and transformed FL often lack a clonal relationship, also

supports the theory of multiple committed precursors that may undergo unique changes creating different malignant clones (64, 76). In support of this model, gene expression profiling has also revealed significantly different expression signatures between low-grade and high-grade FL suggesting that grade 1-2 and grade 3 FL may represent different biological entities (32). Due to the low number of cases, our study was unable to distinguish between grade 3A and grade 3B FL cases, so weather the line of demarcation lies between grade 2 and grade 3A or grade 3A and grade 3B remains to be uncovered.

Finally, the higher number of IGHV mutations in grade 3 FLs enriched for centroblast like large cells as compared to dominance of centrocytes in lower grades of FL was rather unexpected. The V_H gene sequence analysis of microdissected centroblasts and centrocytes from reactive germinal centers showed contrasting features (77). According to previous findings, the centroblasts of the dark zone of a reactive GC tend to express germline-encoded V regions and centrocytes of the light zone of GC express mutated V regions. These data also suggest that the neoplastic centroblasts and centrocytes of FL are not analogous to centroblasts and centrocytes of normal CG at the genetic level.

4. Follicular lymphoma retains the ongoing nature of IGHV mutation during bone marrow involvement suggesting the presence of active hypermutation machinery and the presence of a GC analogous microenvironment in the bone marrow.

During the course of disease progression FL often involves the bone marrow. It has been previously established that the follicle center derived neoplastic centrocytes and centroblasts retain the characteristic ongoing nature of somatic hypermutation of the IGHV gene with lymph node propagation (28-30), however the mutational status characteristics of bone marrow infiltrates was still unknown. In order to shed light onto this question we have compared the IGHV gene mutational status of paired lymph node and bone marrow samples of 3 selected FL cases with known bone marrow involvement as part of a larger study cohort containing 21 untreated FL patients.

Our results demonstrate that the cytological grade of the neoplastic cells and the mutation pattern of IGHV genes are frequently different in the LN when compared to the simultaneous BM infiltrate. The IGHV mutation patterns disclosed significant intraclonal heterogeneity at both of these anatomical locations. With in-depth mutational analysis and through construction of genealogical trees reflecting the intraclonal architecture, we could demonstrate the presence of direct derivatives of the original neoplastic clones from the lymph node as well as newly generated, partially related subclones populating the BM. These findings reveal that the clonal evolution is still ongoing and the mutational machinery is still active in BM involvements of FL. The mutational frequency of clones in the BM and LN are in the same range, however the intraclonal heterogeneity shows only a partial overlap, indicating that the somatic hypermutation activity is independent at these two sites. From the distribution of somatic mutations within the V-regions we can conclude that antigen selection contributes to clonal development at both of these locations. Thus, our results suggest that the BM represents a suitable microenvironment, similar to that of a lymph node, where tumor cells retain the ongoing nature of the somatic mutations of the IGHV genes, and that mutations accumulate in a way that suggests selective pressure by an antigen.

It is widely accepted that the t(14,18) translocation, characterizing the majority of FLs occurs in the prefollicular stage of B-cell development within the bone marrow, parallel with rearrangement of the IgH gene (78, 79). According to the traditional concept of lymphomagenesis, these t(14;18) translocation positive lymphoma progenitor cells - bypassing the natural tumor suppressor mechanisms by overexpression of the anti-apoptotic BCL2 gene - subsequently migrate to the germinal centers of lymph nodes and evolve into follicular lymphoma through a series of acquired aberrant somatic mutations of key differentiation regulatory genes (70, 79-81). Later on, disease progression leads to systemic dissemination and involvement of the bone marrow. The concept of ongoing somatic mutation and independent clonal evolution of BM infiltrates of FL leads to an alternative hypothesis of lymphomagenesis. In this alternative model, the primary malignant transformation of t(14,18) positive progenitor cells may occur in the bone marrow and the lymph node involvement would be the secondary event. In support of this concept, the bi-

directional migration of the neoplastic clones of FL between the lymph node and bone marrow have already been described in the literature (70). Further support for this concept comes from studies showing that FL cells may colonize non-neoplastic germinal centers, and that "*in situ*" FL, often represents infiltration of pre-existing non-neoplastic germinal centers by FL cells, rather than *de novo* formation of malignant germinal centers (28, 82). Furthermore, the quiescence-inducing microenvironment of the bone marrow is known to serve as a protective niche for FL cells, shielding them from the anti-proliferative agents administered with curative intentions, and may provide a base for the progression, persistence and relapse of the disease (83, 84).

5. The progression and transformation of follicular lymphoma is associated with aberrant somatic hypermutation of the BCL6 gene.

Clonal evolution and histological transformation of FL is associated with accumulation of diverse secondary genetic alterations. It has been previously demonstrated that the BCL6 gene can be a target of chromosomal rearrangements and point mutations affecting its 5' noncoding region in a significant proportion of FL and diffuse large cell lymphoma (DLBCL) (52, 85, 86). We analyzed serial biopsy specimens from 12 patients with FL to evaluate the role of BCL6 gene alterations in the histological transformation and clonal progression of FL. Two of our FL showed no histological progression in the second biopsy, while 10/12 cases showed transformation to DLBCL in the second biopsy.

We found no rearrangement of the BCL6 gene in any of the samples, but multiple mutations affecting the 5' noncoding sequences of the BCL6 gene indicating their independence from chromosomal translocations. Seven of twelve cases showed somatic mutations of the BCL6 gene which represented 6 transformed cases and 1 non-transformed case. In the transformed cases, the pattern of BCL6 mutations indicated considerable intraclonal divergence both in the original neoplastic FL clones and also in the transformed DLBCL clones. Comparison of the mutation patterns revealed that histological transformation of FL was associated with the emergence of new subclones with divergent clonotypes of the BCL6 5' noncoding sequences. These findings suggest that the genetic

diversity of the 5' noncoding sequences of the BCL6 gene is generated in the FL or the DLBCL clones and histological transformation is associated with clonotypic shift of the original FL clone.

The nucleotide differences of BCL6 mutations ranged from 2 to 18 between FL and subsequent DLBCL cases. The clonal relationship between the sequential biopsies was confirmed by identical IgH rearrangements. Common origin of FL and DLBCL cells was further supported by the presence of single shared mutations in the original subsequent biopsy samples. The non-shared mutations possibly accumulated during clonal evolution or histological transformation. Somatic mutations of the 5' noncoding region of the BCL6 gene in B-cell NHLs of the GC/post-GC origin has been well documented in the literature (16, 36, 42, 87-89). In the present study, we revealed further characteristics of these mutations showing that BCL6 gene mutations are associated with considerable intraclonal heterogeneity in FL and some heterogeneity in the transformed DLBCL.

The sequence heterogeneity of the tumor clones indicates that the BCL6 gene of the neoplastic cells is still under the influence of the mutation machinery. Evidence for the presence of ongoing somatic mutation the FL clones is not surprising given the fact that these entities derive directly from the germinal centers. On the other hand, intraclonal heterogeneity in the DLBCL transformants was unexpected. These results seem to be in contradiction with our previous findings describing the downregulation of the mutation machinery and loss of ongoing somatic hypermutation activity regarding the IGHV genes with cytological progression of FL and Richter transformation of CLL (34). This discrepancy may point to the differential regulation of somatic mutations of IGHV and BCL6 genes. During the process of high grade transformation, the neoplastic cells may lose contact with their environment (83, 84) leading to decreased stimulation through the B-cell receptor and loss of antigenic exposure that drives IGHV mutations, while the aberrant mutation process targeting non-IgH sequences is still maintained. Another possible explanation is that intraclonal heterogeneity of BCL6 mutations merely reflects of an increased genetic instability, which, in turn, may result in alterations of other oncogenes or tumor suppressor genes responsible for histological transformation of FL.

The functional consequence of BCL6 5' noncoding region mutations is not clear (36, 88, 89). BCL6 expression can be deregulated as a consequence of some of the somatic mutations (16) reflected in altered mRNA expression, while other mutations seem to have no effect on regulation of gene expression (61). Ongoing somatic mutation of the BCL6 5' noncoding sequences have also been documented in normal GC B-cells (61, 90). The pattern of mutations is not clearly different in normal and malignant B-cells, which suggests that the vast majority of these mutations may not have any pathological effect. Several mutations have been detected in the majority of neoplastic clones both before and after the transformation questioning their role in the transformation process. Other BCL6 mutations however, may be selected for by their effect on the survival advantage of the tumoral clone. Some authors describe mutations of the 5' untranslated autoregulatory region of BCL6 resulting in increased BCL6 expression (91, 92). that associates with favorable clinical prognosis (93). Single nucleotide polymorphisms (SNPs) are also a cause of genetic heterogenity at the BCL6 locus. Three SNPs in the 5'UTR have been reported (88), and one appears to be associated with high grade transformation of follicular lymphoma.

The accumulation of mutations in BCL6 gene during high grade transformation of FL, the mutational clustering and the presence of recurrent mutations suggest that certain clones may have been selected for, through intensive clonal selection process, by the favorable effect of these mutations on the survival of the tumoral clone. A mutational variant having selective growth advantage compared to those of parental clones gives rise to the DLBCL population. Thus our results demonstrate functional selection of the neoplastic clones during clonal evolution and histological transformation of FL based on intraclonal heterogeneity of the BCL6 gene associated with ongoing somatic mutations.

5. ORIGINAL CONCLUSIONS

1. Thymic B-cells are follicular/postfollicular cells based on the mutation profile of IGHV and BCL6 genes.

2. The somatic mutation pattern of IGHV gene support postfollicular origin of primary mediastinal B-cell lymphoma.

3. The similar mutational pattern of IGHV and BCL6 genes indicate the common origin of thymic B-cells and primary mediastinal B-cell lymphoma.

4. Low-grade FLs demonstrate ongoing somatic hypermutation in line with follicular origin, while high-grade FLs show lack of intraclonal heterogeneity suggesting termination of ongoing hypermutation compatible with post-follicular like genotype.

5. Follicular lymphoma retains the ongoing nature of IGHV mutation during bone marrow involvement suggesting the presence of active hypermutation machinery and the presence of a GC analogous suitable microenvironment in the bone marrow.

6. The progression and transformation of follicular lymphoma may be associated with aberrant somatic hypermutation of the BCL6 gene.

6. SUMMARY

The analysis IGHV gene has come a long way since it was first described in the 1980's along with the discovery of the genomic structure of the immunoglobulin heavy chain gene locus (94). At its hay-day in the 1990's demonstration of the clonal IgH rearrangement was about the only objective tool to assess malignancy. Later on, as further details about IgH gene processing accumulated, it was started to be utilized for establishment of cellular origin, which provided useful information for molecular lymphoma classification. Another milestone of IGHV analysis was the description of the binomial distribution model (38), that allowed demonstration of genomic footprints of antigen exposure. Years later, as larger scale sequencing became routine laboratory practice, the determination of intraclonal heterogeneity by subclone sequencing was another useful step to study clonal evolution to gain better understanding about the mechanism of lymphoma progression. Before the first decade of the 21st century the sequence analysis of IGHV gene was mostly utilized for basic research purposes. Nowadays as high throughput sequencing is widely available the previously accumulated knowledge is being translated into the everyday routine diagnostic algorithms, such as CLL prognostication and treatment stratification based on mutated/unmutated IGHV status. In my studies, I attempted to provide brief examples for useful applications of IGHV pattern analysis.

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8. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

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