Immunoglobulin gene alterations in the progression of B cell lymphomas

The studies described in this thesis were performed at the Department of Pathology, Academic Medical Center (AMC), University of Amsterdam, The Netherlands.

The cover:

Expression of Activation Induced Cytidine Deaminase in human germinal centre B cells. This immunohistochemical staining performed on a human tonsil clearly shows the architecture of a typical germinal centre with an AID-positive dark zone, light zone and an AID–negative mantle zone. The immunohistochemical staining was optimized and performed by Thera Wormhout and Febe van Maldegem.

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Immunoglobulin gene alterations in the progression of B cell lymphomas

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1. General Introduction

The innate and adaptive immune response

Humans have several lines of defense to protect them against the huge repertoire of microorganisms that may cause disease. As soon as a pathogen crosses an epithelial barrier it can immediately, without prior infection, be recognized by cells of the innate immune system like granulocytes, macrophages, mast cells and natural killer (NK) cells [1]. This will lead to the secretion of cytokines and other inflammatory mediators and to phagocytosis and destruction of the invading pathogen. Macrophages can function as professional antigen-presenting cells (APC) and are as such important in the induction of the adaptive immune response[1]. Pathogen recognition by dendritic cells is mediated by Toll-like receptors (TLR) that are expressed on the cell surface. Signaling via TLR is essential for dendritic cell maturation, the expression of co-stimulatory molecules like CD80 and/or CD86 and thus for priming of naïve T-cells [2]. Whereas the innate immune response directed against a new antigen is swift, the adaptive immune response requires the clonal selection of B and T lymphocytes, which takes several days to accomplish [3]. The antigen-driven adaptive immune response can be divided in a T-cell mediated cellular branch and an antibody mediated humoral branch and provides immunological memory. Re-encounter with the same pathogen will then lead to a quicker and stronger immune response.

1.1 Antigen independent B-cell differentiation

B-cells are generated out of haematopoietic stem cells in the bone marrow throughout life. Although early B-cell development in humans is not as extensively investigated as in mice, several differentiation stages have been identified (Figure 1)[4-6]. The common lymphoid progenitor (CLP) is the first cell that shows lineage-commitment. *In vitro* experiments showed that stimulation of mice CLP induces differentiation into lymphoid cells (B-cells, T-cells, Natural killer cells and dendritic cells) but no longer into myeloid or erythroid cells[6;7]. Knock-down experiments in mice showed that differentiation of the CLP into pro-B cells is critically dependent on the transcription factors E2A, EBF and PAX5 [5;7]. E2A and EBF act synergistically to induce the expression of PAX5 and thus B-cell specific genes like RAG1, RAG2, TdT, mb-1 (Igα) and the surrogate light chain genes λ5 and VpreB [8]. It is postulated that differentiation of CLP into pro-B-cells goes via an early B-cell intermediate. The CD19-negative early B-cells are characterized by the RAG-induced ordered rearrangement of *D* and J_H gene segments at the immunoglobulin heavy chain (*IgH*) locus [4]. *D*- J_H rearrangement can be detected in later differentiation stages as well [9]. In pro-B cells V_H to DJ_H rearrangement occurs [10;11]. Of note, other reports describe that V_H-DJ_H transcripts are not detectable until the pre-BI stage [9]. LeBien reports that a functional V_HDJ_H rearrangement is the prerequisite for differentiation into a pre-BI cell [4]. At this stage of B-cell differentiation, the rearranged *Ig* heavy chain combines with the invariant surrogate light chains VpreB and λ5 to form a pre-

BCR [12]. Surface expression of a pre-BCR acts as a key checkpoint for further differentiation. The pre-BCR$^+$ cells downregulate RAG1 and RAG2 and undergo several rounds of proliferation [6]. In the pre-BII cells, RAG1 and RAG2 are re-expressed for recombination of the immunoglobulin light chain (*IgL*) genes. After successful V_L to J_L rearrangement of the *Igκ* and/or *Igλ* locus, the light chain covalently binds to the *Ig μ* heavy chain and forms a BCR [4;6]. B-cells that fail to produce a functional BCR will undergo apoptosis. Furthermore, experiments using transgenic mice showed that cross-linking of the BCR from immature B cells can have different outcomes. High-affinity interactions with membrane-bound antigen lead to clonal deletion of the particular B cell whereas lower affinity interactions with soluble antigen either makes the B-cell non-responsive (anergic) or will result in rearrangement of the BCR (receptor editing) in order to prevent migration of auto-reactive B cells into the periphery [7].

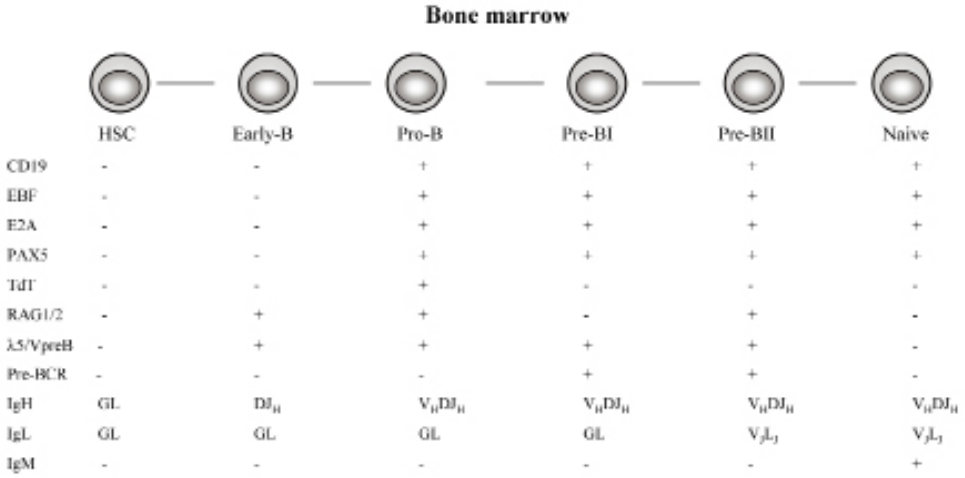

Figure 1: Human early B-cell development. Schematic representation of human B-cell differentiation stages in the bone marrow and the expression of components of the (pre-)BCR, RAG1, RAG2 and several transcription factors. GL; germline configuration, HSC; haematopoietic stem cell [4;6;9].

1.2 Antigen-dependent B-cell differentiation

B-cells can be activated in a T-cell independent manner by bacterial polysaccharides and other microorganism-derived Toll-like receptor ligands, which results in a rapid antibody response through the generation of extra-follicular short-lived plasma cells. Alternatively, within the secondary lymphoid organs B-cells can engage in a T-cell dependent immune response leading to differentiation into memory B cells and long-lived plasma cells to produce high-affinity antibodies.

There are three subsets of mature B cells in mice; B1 cells, marginal zone B cells and follicular B cells. These B cells differ from each other in their characteristic distribution

throughout the lymphoid system, their surface phenotype and their propensity to engage in a T-cell dependent or independent immune response. In humans $CD5^+$ B-cells that share other phenotypic characteristics with murine B1 exist, but a human B1 population has as yet not been defined. Antibodies produced by B1 cells show reactivity with common bacterial antigens and self-antigens. MZ B cells share the capacity with B1 cells to be involved in the rapid T-cell independent immune response raised against blood-borne antigens [13]. MZ B-cells and B1 cells both have self-renewal capacity whereas follicular B cells continuously develop from progenitor cells in the bone marrow. Recirculating naïve follicular B cells can encounter blood-borne antigens in the bone marrow [14] and spleen and can there participate in the T-cell independent immune response, or alternatively can be activated by antigen presented by APC in secondary lymphoid tissues which requires T cell help [15]. Homing of the naïve B cells into the primary follicles in lymph nodes and spleen is mediated by the CXC receptor 5 (CXCR5) and its ligand CXCL13 (BLC) which is expressed by FDC and other, less differentiated, stromal cells[16].

1.3 The germinal center

Germinal centers are specialized microenvironments within secondary lymphoid organs that are essential for the generation of B-cells that produce high affinity antibodies of different isotypes and for the generation of memory B-cells [1]. Naïve B cells, which are $IgM^+IgD^+CD38^-CD23^+$ or $CD23^-$ differentiate into GC B cells, which are $IgD^-CD38^+CD10^+CD71^+$ via recently discovered pro-GC $IgD^+CD38^-CD10^-CD71^+CD23^-$ and pre-GC $IgM^+IgD^+CD38^+$ subpopulations [17]. Germinal centre B-cells are further characterized by the expression of the B-cell specific transcription factor BCL6 and Activation induced cytidine deaminase (AID)[18]. Histologically, a fully developed germinal center can be divided into two compartments: the dark zone and the light zone. Several chemokines have been described to play a role in the formation these compartments. Experiments in knock-out mice showed that the interaction between CXCR4, which is highly expressed by centroblasts and its ligand SDF1/CXCL12 is essential for GC dark zone formation. whereas the interaction between CXCR5 and its ligand CXCL13 is important in light zone localization [16].

Cross-linking the BCR of naïve B cells in combination with accessory signals provided by T-cells can result in either direct differentiation of the B-cell into an IgM secreting plasma cell, or alternatively, participation in the germinal centre reaction [19]. Due to the upregulation of CCR7, a shift in the balance of chemokine responsiveness and re-localization of the B cells from the primary follicle to the B/T-zone boundary occurs. After processing and presentation of the antigen in MHC class II on the B cells, interaction of a T-cell receptor (TCR) with MHC results in activation and clonal expansion of the antigen-specific T cells. This process is dependent on co-stimulatory signals derived from CD28-CD86 interactions. On their

turn, primed antigen-specific $CD4^+$ T cells express CD40L and CD28/CD152 which will activate CD40, CD80 and CD86 on Ag-activated B-cells, resulting in the initiation of the germinal centre reaction [15]. The rapid proliferation of the $CD77^+$ GC centroblasts, does not seems to be driven by C-MYC or NFκB [18]. But the molecules produced and presented by FDCs, such as IL-15, 8D6 (CD320), BAFF and hepatocyte growth factor (HGF), may have a role in centroblast proliferation and/or survival [20-24]. The proliferating centroblasts push the IgM^+IgD^+ naïve B cells from the primary follicle aside, which then forms the follicular mantle zone. It now seems clear that not only antigen-specific naïve B cells but also memory B cells can enter the GC reaction [25;26].

Centroblasts express low amounts of surface BCR (sBCR) and undergo somatic hypermutation of their variable-region (IgV_H) gene segments [27]. The process of somatic hypermutation introduces mostly point mutations, which will alter the affinity of the BCR for its antigenic epitope. Centroblasts then exit the cell cycle, re-express their sBCR and migrate to the light zone of the GC to become centrocytes. Here, they are in close contact with a network of FDC, expressing the FcγR-IIB, complement receptors such as the long splice variant of CD21 and CD35, and the adhesion molecules VCAM and ICAM [28]. FDC are specialized antigen-presenting cells and competition of the B-cells for the presented antigen will result in the survival of those centrocytes expressing BCRs with the highest affinity for the antigen, a process commonly referred to as affinity maturation. B cells that express a non-functional BCR or a low-affinity BCR will undergo programmed cell death, called apoptosis [3]. Two-photon microscopy in mice showed bi-directional migration between the dark and light zones, suggesting that multiple rounds of proliferation and selection take place [16;26]. Next to the FDC, CD40L-expressing T cells present in the light zone also play an important role in the centrocyte selection. Blocking antibodies against CD40L can disrupt established GC in mice. Mutations in the genes encoding either CD40 or CD40L result in an immunodeficiency syndrome called the hyper-IgM syndrome (HIGM1), which is characterized by the impairment to switch Ig isotypes, and the absence of GC and memory B-cells [29].

In addition to SHM, a second IG diversification process takes place: class switch recombination (CSR). CSR occurs within the GC light zones and involves the coordinated deletion of *Ig* constant region genes what will result in the expression of a different Ig isotype. Switching of the constant region alters the effector function of the antibody without altering its antigenic specificity. The constant region determines the biological activity of the antibody and determines how antigens are removed from the body. There are several constant region genes i.e. μ, δ, γ, ε and α encoding IgM, IgD, IgG, IgA and IgE antibodies respectively, each specialized in the clearance of antigens in specific body compartments. In short, IgM antibodies form pentamers, are mainly present in the blood and are very

potent complement activators. IgG antibodies are present in the blood and in the tissues, can opsonize pathogens and bind Fcγ-receptors on phagocytic cells. IgA antibodies are the principal antibodies present in secretions, like gastro-intestinal fluids and tears. They are less potent activators of complement or phagocytic cells and function mainly as neutralizing antibodies. Finally IgE antibodies provide protection against parasitic nematodes, and play an important role in allergy [1]. Class switching is initiated by cytokine-induced transcription of the switch regions that are located upstream of the constant region genes, called sterile or germline transcription. Germline transcripts are further processed by RNA splicing that fuses the I exon to the C exon thereby deleting the switch region. Both germline transcription and the subsequent splicing of the germline transcripts are required for CSR [30]. In vitro analysis revealed that when transcription of the highly repetitive guanine-rich switch regions occurs, the transcribed RNA hybridizes with the template DNA to form a stable DNA-RNA hybrid, termed an R-loop, in which the non-templated DNA strand is looped out as single-stranded DNA (ssDNA) [31]. ssDNA can be targeted by AID, the enzyme essential in both SHM and CSR (see below) [32-34]. Switch regions are rich in RGYW motifs (where R denotes an A or G, Y denotes an C or T and W denotes an A or an T) which are preferential target sites of AID[35]. During CSR double stranded DNA breaks are observed in the switch regions, but how exactly AID can target the opposing strand to create the double stranded nick is not quite clear yet [36]. Substituting mice switch regions with *Xenopus* switch regions, which forms no R-loops, but does contain palindromic AGCT sequences, (a subset of the RGYW motif) still resulted is CSR in vivo, indicating that CSR can also occur via an R-loop independent mechanism [37]. The joining of the two cleaved switch regions is mediated by non-homologous end joining (NHEJ) and results in looping out of the intervening DNA and the expression of a different constant region gene [30].

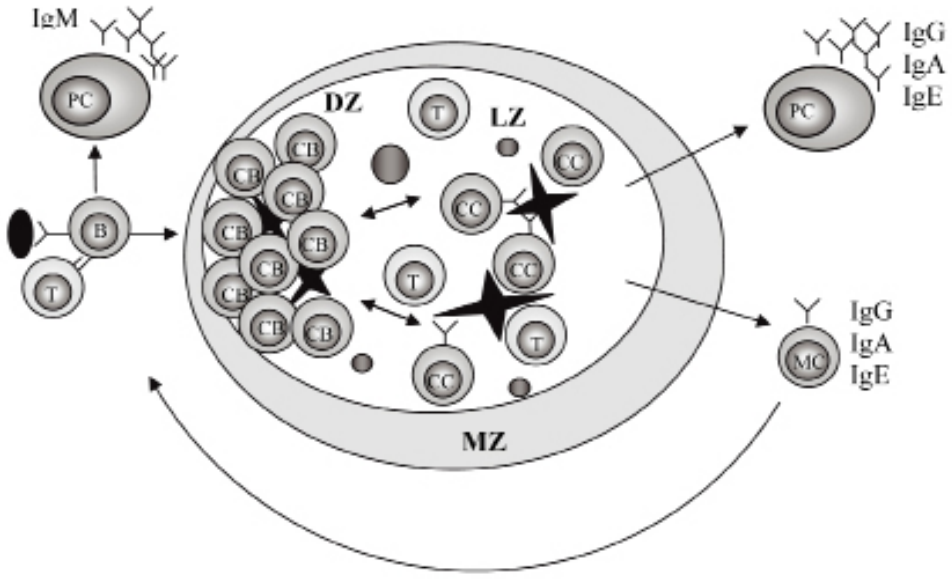

Figure 2: The germinal centre reaction. The interaction of antigen-specific naïve or memory B cells with antigen specific T-cells can result in either direct differentiation into an IgM-producing plasma cell or the initiation of the GC reaction. $CD77^{+}$ centroblasts proliferate rapidly within the GC dark zone, thereby pushing aside the naïve B-cells, which then form the GC mantle zone. Within the GC light zone, antigen presented by FDCs (black stars) and CD40L expressed by T-cells are essential for the process of affinity maturation. Massive apoptosis occurs (small grey circles). Only the centrocyte that express a high affinity, antibody will differentiate into either a plasma cell of memory cell. Multiple rounds of proliferation and selection can take place as indicated by the curved arrow. B; B cell, CB; centroblast, CC; centrocyte, DZ; dark zone, M; memory B cell, MZ; mantle zone, PC; plasma cell, LZ; light zoneT; T cell.

1.4 Somatic hypermutation

The process of SHM has been shown to exhibit several characteristics [38]. SHM is restricted to a 1-2kb region downstream of the IgV_H promoter, which results in the specific targeting of IgV_H but protects the constant region. Furthermore, the amount of IgV_H mutations is proportional to the transcription rate of that locus. SHM occurs at a rate of 10^{-3} mutations per base pair per cell division and introduces mostly point mutations into the IgV_H genes, although insertions and deletions of single nucleotides have been described as well. Specific motifs in which mutations occur more often are the RGYW motif or its reverse complement WRCH (where R denotes an A or G and H denotes an T, C or A) [39;40]. Transitions (purine (G and A) to purine and pyrimidine (C and T) to pyrimidine) occur twice as frequent as transversions (purine to pyrimidine and vice versa) and there is a clear strand bias [41]. Considerable efforts have been made to address this issue of targeting of the SHM machinery. Duplication of the Ig promoter upstream of an *IG*κ constant region transgene resulted in SHM of this constant region, which

suggested a role for the promoter in the targeting of the SHM machinery [42]. In addition, substitution of the IgV_H gene with a variety of sequences all lead to SHM of the sequence located between the V promoter and an intronic enhancer [43]. However, replacement of the V promoter by the β-globin promoter still showed mutations in the IgV_H gene [44] indicating that the targeting does not require sequence specific motifs within the IgV_H promotor. Several papers have outlined the importance of IGH and IGκ enhancers for the targeting of the SHM machinery although the results are conflicting [45]. In SP6 hybridoma cell lines deletion of the core Eμ enhancer severely diminished SHM, as did deletion of its flanking matrix attachment regions (MAR). However, deletion of Eμ together with its MAR restored SHM, and in Eμ knock-out mice SHM was completely unaffected, which indicates that these elements exert both positive and negative regulative properties [43]. Finally with the discovery of the enzyme AID a new set of data became available. It became clear that overexpression of this enzyme in B-cells and non B-cells induced SHM in non-Ig genes, suggesting that the targeting of SHM can occur even without the presence of Ig regulatory elements [35;46-48].

Epigenetic alterations have been implicated in the targeting of SHM as well. Histone acetylation of the IgV_H region has been described to be correlated with SHM in the Burkitt lymphoma cell line BL2 [49]. Contradictory, *in vivo* experiments using *IgH* transgenic mice showed that H3 and H4 acetylation of the endogenous *V*λ gene was similar to that of the *C*λ gene, both in naïve and GC cells. Within the same mouse model phosphorylation of histone 2B tightly correlated with both SHM and CSR [50]. In addition, methylation of CpG repeats seemed to protect ssDNA *in vitro* against AID-mediated deamination [51].

1.5 Activation-induced cytidine deaminase

In the human genome *Aicd* is located at 12p13, it is composed of 5 exons and encodes a 24 kD protein that consists of 198 amino acids [52]. The AID protein has the highest sequence homology to the RNA editing enzyme apolipprotein B mRNA editing catalytic polypeptide 1 (APOBEC-1). Based on this homology, several structural and functional domains have been assigned. The N-terminal part of the protein is composed of a helix domain with a nuclear localization signal (NLS) [53]. Although this NLS has not been identified by all groups[54], mutations in this region result in confinement of AID to the cytoplasm and a complete loss of its function [53]. The active site of the protein also at N terminal part of the protein, contains a cytidine deaminase motif [55] and the amino acids essential for dimerization of the protein, which has been shown to be essential for its function [56]. The C-terminal part of the protein is composed of an APOBEC-like domain that contains the pseudoactive site and an nuclear export signal [53;54]. A three dimensional structure of AID is predicted based on crystallographic analysis of yeast cytosine deaminase D1 but the crystal structure of AID itself is not published yet [57].

AID was first identified after subtraction of cDNA libraries from a CSR-induced and non-induced B-cell lymphoma cell line [58]. Targeted deletion of AID was shown to abolish both SHM and CSR in mice [59], and gene conversion in chicken [60]. Lack of intact AID expression in humans leads to the hyper-IgM syndrome 2 (HIGM2), a rare immunodeficiency characterized by elevated serum IgM levels with absence of IgG, IgA or IgE, the lack of IgV_H mutations and an increased susceptibility for infections [61]. Enforced expression of AID in fibroblasts [47] and bacteria [33] confirmed its essential role in SHM and CSR. Mutations introduced in these *in vitro* experiments exhibited the same key features (such as a preference for point mutations, transitions over transversions, the strand bias and specific targeting of certain nucleotide motifs) as those found *in vivo* in mice and men [33;35;47].

In accordance with its function in SHM and CSR, AID is expressed by GC B-cells, but also by scattered interfollicular large B-cells and single B-cells throughout the T-cell zone and mantle zones [62]. AID expression can be induced *in vitro* in human B-cells by IL4 and CD40L. Several transcription factors are now known to regulate the expression of AID, in particular PAX5 and E47 [63]. A putative enhancer sequence has been described 24 kb 3' of human *Aicd*, which is essential for AID transcription [64].

The high sequence homology of AID to the RNA-editing enzyme APOBEC-1 initially led to the hypothesis that AID is an RNA editing enzyme [58]. This was supported by the findings that AID possesses cytidine deamination activity just like APOBEC-1, and that both genes are closely mapped on human and mice chromosomes. *In vitro* experiments showed that AID can bind both RNA and ssDNA [32-34;65;66]. However, ectopic expression of AID in E. coli induced widespread mutations and since it is rather unlikely that humans and bacteria share the same mRNA substrates, AID is now considered to be a DNA mutator [48]. Indeed, AID-mediated mutations are enhanced by a deficiency of uracil DNA glycosylase (UNG), and this has led to the following DNA deamination model (Figure 2). Deamination of a cytidine by AID turns it to a U (uracil). Direct replication of the DNA U - G mismatch will lead to C→T or G→A transitions in 50% of the daughter cells. However, when the U-G mismatch is recognized by the base-excision repair (BER) system, the uracil will be removed by the enzyme uracil-DNA glycosylase (UNG). The remaining abasic site will be cleaved by an endonuclease and normally a C will be reinserted. However, when DNA synthesis occurs, an error prone polymerase can insert any nucleotide at the abasic site. Alternatively the U-G mismatch can also be recognized by the mismatch repair (MMR) machinery. A single stranded DNA gap is then created around the U-G mismatch, followed by "patch" repair by a translesion DNA polymerase, resulting in A/T biased mismatches in the close vicinity of the previous U-G mismatch [67;68].

According to this model, cleavage by the endonuclease may result in (temporary) DNA breaks. Indeed double stranded DNA breaks (DSB) have been described in RGYW motifs of

IgV_H genes in B cells undergoing SHM [69;70]. Of note, DSB have been described with the same frequency and distribution in AID deficient cells, indicating that these breaks can also occur independent of the process of SHM [71].

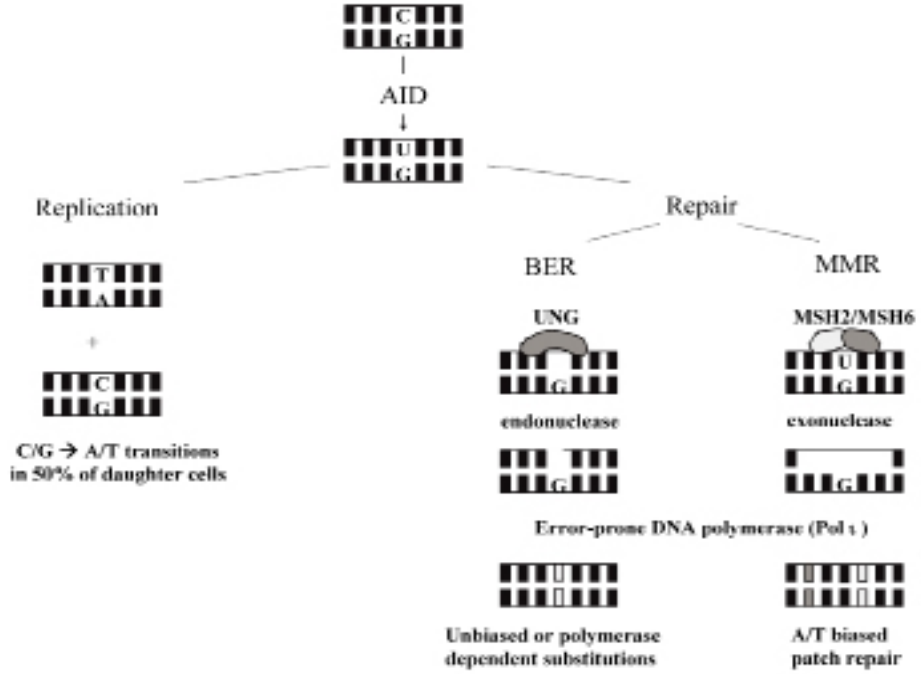

Figure 3: AID in somatic hypermutation. DNA deamination model according to Neuberger *et al* [67;68]. AID deaminates a cytidine to a uracil. When direct replication takes place this will lead to a C→T or G→A transition in 50% of the daughter cells. Excision of the uracil by the BER will lead to unbiased or polymerase biased substitutions whereas repair by the MMR pathway will lead to mutations of and around the original U/G mismatch. White and grey rectangles indicate mutated nucleotides. BER; Base excision repair, MMR; mismatch repair, UNG; Uracil DNA glycosylase, MSH2/MSH6; Mut S homologue 2 and 6.

Analysis of the AID gene of HIGM2 patients and other AID mutants showed that alterations in the N-terminal region impaired SHM without affecting CSR [72], whereas alterations in the C-terminal region abrogated CSR while leaving SHM activity intact [54]. This indicates that different co-factors are necessary for execution of SHM and CSR. AID from activated B-cells is phosphorylated by protein kinase A (PKA) and this phosphorylation is essential for the interaction of AID with the co-factor Replication Protein A (RPA) [73]. RPA is a ssDNA-binding protein involved in DNA replication, recombination and repair [74]. Although its exact role in SHM is not completely clarified, it is hypothesized that the AID-RPA complex binds to and stabilizes ssDNA at small transcription bubbles. In addition, RPA may act as a scaffolding protein for the entry of DNA repair proteins, such as members of the mismatch repair machinery and BER, like UNG. *In vitro* binding studies revealed that in contrast to

typical transcription bubbles which are 14-17 nucleotides in size, AID binds preferentially 5-9 nucleotide bubbles, such as stem loop bubbles which are created during transcription or replication of DNA due to local unwinding of the DNA [75]. Interestingly, this study also showed that although the cytidine deaminase event is sequence (WRC) specific, AID itself binds the ssDNA transcription bubbles irrespective of its sequence, which means there are other factors involved in the targeting of SHM. Another co-factor that has been described to interact with AID is MDM2, a ligase that is involved in cytoplasmic p53 degradation, but the role of this interaction in the process of SHM is not yet entirely understood [36].

1.6 Apoptosis

During the GC reaction only B cells expressing high-affinity antibodies that are not self reactive will be selected to survive. Therefore, the majority of the B-cells will undergo programmed cell death, also called apoptosis. Apoptosis is executed by the activation of caspases. There are two major pathways that can activate caspases; the extrinsic and intrinsic pathway [76;77].

The extrinsic pathway is induced upon activation of receptors belonging to the TNFR-super family, such as Fas/CD95. A death inducing signaling complex (DISC) is then formed in which the adaptor protein Fas-associated-death domain containing protein (FADD) bind to the CD95 receptor, which causes the aggregation and activation of procaspases 8 or 10. Signaling via the death receptors can be inhibited by FLICE-inhibitory proteins (FLIP)[78]. Autocatalytic cleavage of procaspase 8 results in activation of effector caspases, which carry out proteolysis on numerous vital and structural proteins and thus cause the controlled demolition of the cell. A positive amplification loop is formed by the activation of initiator caspases by the effector caspases. Caspase 8 can also engage the intrinsic apoptosis pathway by activating the pro-apoptotic BH3-interacting-domain death agonist (BID)[79].

Cytokine deprivation, hypoxia, DNA damage and cytotoxic drugs induce apoptosis via the intrinsic pathway, also called the mitochondrial pathway. Both pro-and anti-apoptotic BCL2 family members control the release of mitochondrial cytochrome c via proapoptotic BCL2 associated X protein (BAX)- and BCL2 antagonist/killer (BAK)– mediated disruption of the mitochondrial membrane. Cytochrome C binds to the scaffolding protein apoptotic protease-activating factor 1 (APAF-1). Binding of caspase 9 to Apaf-1 oligomers result in the formation of the apoptosome and this will activate the effector caspases, again leading to cell death. Crucial in the regulation of the intrinsic pathway are the BCL2 family members, which can be recognized by their BCL2 homology (BH) domains. Based on their structure and function three BCL2 subfamilies can be identified. The anti-apoptotic BCL2 family members, like BCL2, BCL-xL, BCL-w, Mcl1 and A1/Bfl-1, the pro-apoptotic BAX/BAK-like proteins such as BAX, BAK, BCL-xs and BOK and the pro-apoptotic BH3-only family members like Bad,

Bik, Bid, Bim, Hrk, Noxa and Puma. The BH3-only proteins exert their function by binding to the anti-apoptotic BCL2-like proteins and require members of the BAX/BAK like subgroup to trigger apoptosis. However, it is as yet not entirely understood how the interaction between the BCL2-like proteins, the BAK/BAX like proteins and the BH3-only proteins control cell death [77]. Another group of proteins that regulate apoptosis are the inhibitors of apoptosis (IAP), including XIAP, IAP1, IAP2, Survivin and Livin. These anti-apoptotic proteins act downstream of the mitochondria by binding to caspases as competitive inhibitors. IAPs are neutralized by mitochondrial proteins which can bind and sequester them [80].

Germinal centre B-cells express high levels of the apoptogenic CD95, Bax, the BH3-only gene BIK and the proto-oncogene p53 [18;81;82]. In addition, despite the CD40 stimulation within the GC microenvironment, microarray analysis showed a lack of a NFκB signature [83;84] in combination with absence of the anti-apoptotic proteins BCL2 and TOSO [18]. Altogether GC B-cells show a gene expression profile in favor of apoptosis. In addition, immunoprecipitation of freshly isolated GC B cells showed the presence of a preformed DISC complex which included FAS, FADD and pro-caspase 8. However, FAS-mediated apoptosis is thought to be prevented by the simultaneous presence of anti-apoptogenic caspase 8 inhibitor c-FLIP$_L$ in this complex [85]. *In vitro* culturing of unstimulated GC B cells result in the rapid decay of c-FLIP$_L$ and the induction of apoptosis. This can be counteracted at least *in vitro*, but probably also in vivo in the GC-microenvironment, by BCR-, CD40- and FDC-mediated stimulation, which result in sustained expression of c-FLIP$_L$ and the prevention of apoptosis [85;86].

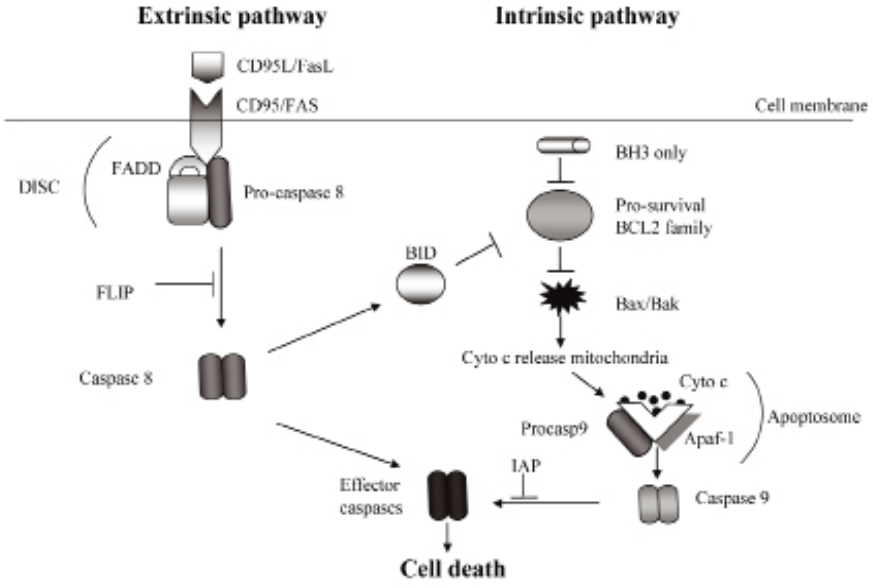

Figure 4: The extrinsic and intrinsic apoptosis pathways. Two pathways that will lead to the activation of effector caspases and apoptosis. See text for details. Adapted from Marsden and Strasser ARI 2003[76;77]

2. B-cell malignancies

Similar to normal B cell differentiation stages, distinct subsets of B-cell non-Hodgkin's lymphomas (B-NHL) can be identified on the basis of 1) the configuration of the BCR, 2) expression patterns of differentiation markers and 3) morphology. Based on these features B-NHL seem trapped at particular stages of B-cell development (Figure 5). This can be explained by oncogenic alterations that can interfere with differentiation of the malignant B-cell. For example, in diffuse large B-cell lymphomas (DLBCL) promoter substitution of the transcriptional repressor BCL6 by the *IgH* promoter leads to continuous repression of a crucial regulator of plasma cell differentiation, B-lymphocyte-induced maturation protein 1 (BLIMP1). In addition, the malignant B-cells become less responsive for external clues that regulate normal B-cell differentiation.

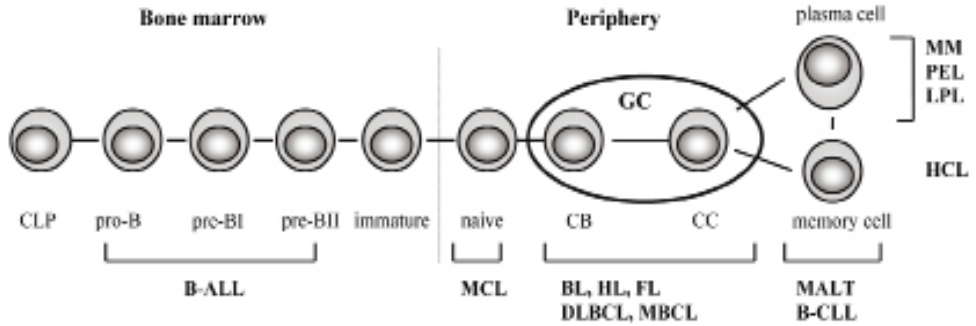

Figure 5: B-cell differentiation and B cell neoplasia. Schematic representation of the different B-cell differentiation stages in the bone marrow and periphery and their malignant counterparts. B-ALL; precursor B lymphoblastic leukemia, B-CLL; chronic lymhocytic leukemia, CLP; common lymphoid progenitor, DLBCL; diffuse large B cell lymphoma, FL; follicular lymphoma, HCL; hairy cell leukemia, LPL; lymphoplasmacytic lymphoma, MALT; marginal zone B cell lymphoma of mucosa-associated lymphoid tissue, MBCL; mediastinal large B-cell lymphoma, MCL; mantle cell lymphoma, MM; multiple myeloma, PEL; primary effusion lymphoma.

The majority of the NHL are of B-cell origin and show a (post-)GC phenotype. Many B-NHL contain balanced chromosomal translocations involving the *Ig* locus and a proto-oncogene (Table 1). As a result cell cycle regulators (like Cyclin D1 in mantle cell lymphomas), transcription factors (like BCL6 in DLBCL and C-MYC in Burkitt's lymphomas) or apoptosis regulating genes (like BCL2 in follicular lymphomas) are placed under the control of the *Ig* enhancers, and this is thought to be the initiating step in B-cell lymphomagenesis. These translocations are thought to occur during the formation and revision of the BCR. In pre-GC tumors, like mantle cell lymphoma (MCL), and some GC-tumors like follicular lymphoma (FL) the *Ig* translocation breakpoints are located adjacent to the $IgV_H D$ and J_H gene segments within specific recombination signal sequences (RSS), consistent with the occurrence during V_H-D-J_H rearrangement. Indeed, RAG-mediated DNA cleavage is responsible for the DNA

breaks in both the *Ig*- and the *BCL2* locus in FL [87]. In some other GC-tumors, like endemic Burkitt's lymphomas (BL) the *Ig* breakpoint is within the V_H exon, the region in which SHM takes place. Finally, in many other GC- and post-GC tumors like sporadic BL, DLBCL and Multiple Myeloma (MM) the breakpoint lies within the *Ig* switch region suggestive for the occurrence during CSR.

		Translocation		SHM	
Lymphoma	**Frequency**	**Locus**	**Genes**	**IgVH**	**ASHM**
MCL	6%	t(11;14)	BCL1 -IgH	30%	nd
BL	3%	t(8;14)	IgH - MYC	+	30%
		t(2;8)	MYC -Igκ		
		t(8;22)	MYC -Igλ		
HL	10%	-	-	+	55-80%
FL	22%	t(14;18)	IgH - BCL2	+	30%
		t(3;14)	BCL6 -IgH		
DLBCL	31%		BCL6 - various	+	47 - 100%
		t(14;18)	BCL2 -IgH		
		t(8;14)	IgH - MYC		
		t(8;22)	MYC -Igλ		
		t(3;14)	FOXP1 -IgH		
MBCL	2%	-	-	+	74%
MZBCL	7%	t(11;18)	API2 -MALT1	+	76%
		t(1;14)	BCL10 -IgH		
		t(14;18)	MALT1 -IgH		
		t(3;14)	FOXP1 -IgH		
		t(11;14)	BCL1 -IgH		
B-CLL	6%	-	-	50%	0%
HCL	<1%	-	-	+	nd
PEL	<1%	-	-	+	66%
MM	10%	t(11;14)	BCL1 - IgH	+	nd
		t(4;14)	FGFR3 -IgH		
		t(14;16)	C-MAF- IgH		
		t(6;14)	CyclinD3- IgH		
LPL	2%	t(9;14)	PAX5 -IgH	+	nd

Table 1: Mature human B-cell lymphomas, translocations and somatic hypermutation.
In the column of ASHM the percentage of lymphomas that shows mutations in one of the following genes is described: PAX5, TTF/Rho, PIM1 or C-Myc. The variability of ASHM in DLBCL is due to differences between different studies. The variability of ASHM in HL is due to differences between HL subtypes with 80% ASHM of lymphocyte predominant HL and 55% ASHM of classic HL. ASHM; aberrant somatic hypermutation, BL; Burkitt's lymphoma, B-CLL; chronic lymhocytic leukemia, DLBCL; diffuse large B cell lymphoma, FL; follicular lymphoma, HCL; hairy cell leukemia, HL; Hodgkin's lymphoma, LPL; lymphoplasmacytic lymphoma, MZBCL; marginal zone B cell lymphoma, MBCL; mediastinal large B-cell lymphoma, MCL; mantle cell lymphoma, MM; multiple myeloma, PEL; primary effusion lymphoma.

Although chromosomal translocations that are a result of aberrantly controlled *Ig* remodeling processes are considered to be a hallmark of B-NHL, other transforming events have also been

described. First, genetic alterations, like mutations (TP53, ATM and CD95), amplifications (REL), deletions and translocations (API2-MALT1) can also occur outside the *Ig* loci. [88;89]. Second, chronic stimulation of the immune system either due to infectious pathogens like *H. pylori* and Hepatitis C virus or due to organ-specific auto-immunity like Sjögren sialadenitis or Hashimoto's thyreoïditis may contribute to MZBCL lymphomagenesis. Third, other viruses may also have oncogenic potential in B-cells, like Epstein Barr virus (EBV) in BL and HL, and in primary effusion lymphomas next to EBV also human herpes virus 8.

2.1 The antigen receptor in B cell non-Hodgkin's lymphomas

Despite the fact that the most of the B-NHL have a chromosomal translocation involving the *Ig* locus, they still express a BCR. Most of these *Ig* translocations are found on the non-productive allele. The great majority of B-NHL shows a GC- or post-GC phenotype with somatically mutated *Ig* genes. Mutation analysis revealed that, like normal B-cells, there is counterselection for replacement mutations in the framework region of the *Ig*. In contrast, higher ratios of replacement versus silent mutations were found in the antigen binding site of the *Ig*, the complementary determining regions (CDR). Even in B-NHL in which ongoing somatic hypermutation has taken place during malignant growth, similar mutational patterns have been observed. Altogether, this implies that lymphoma cells, like normal B-cells undergo stringent selection for the expression of a functional BCR.

In addition to the fact that FL have a structurally intact BCR, they grow within networks of non-malignant FDCs, strongly suggesting that they are still dependent on antigen contact for their survival [90]. Similarly, MALT lymphomas can infiltrate and colonize reactive B-cell follicles. Structural analysis of the antigen receptor in an elaborate panel of B-NHL showed that B-CLL expressed restricted CDR3 sequences. Part of the B-CLL express Ig that show reactivity for auto-antigens like cardiolipin, myoglobulin and rheumatoid factor[91]. In addition, as mentioned above, MALT lymphomas arise at sites of chronic inflammation. Taken together, most B-NHL not only express a structurally intact BCR, it also seems that at least in part of them the BCR is functional, which implies that these lymphomas are still critically dependent on BCR-derived survival signals.

2.2 AID and lymphomagenesis

The IgV_H genes are not the only genes that can be targeted by the somatic hypermutation machinery. Other genes, like BCL6 and CD95 are described to be substrates as well in non-malignant B cells. Furthermore, in a proportion of the DLBCL *C-MYC*, *PAX5*, *PIM1* and *RHO1* carry SHM-like mutations. Aberrant SHM (ASHM) not only affects DLBCL. The four genes targeted by ASHM have different functions, but are all proto-oncogenes. The mutations in *C-MYC* are located around the 5'regulatory region and may affect gene

transcription. Furthermore, ASHM has been described to introduce a stop codon in *C-MYC*, to mutate a start codon in *PAX5* and to introduce amino acid substitutions in *PIM1* and *C-MYC*. ASHM may thus interfere with the protein expression and function as has been described for BCL6 [92]. In addition, all these proto-oncogenes are preferred translocation partners of the *Ig* genes in DLBCL and the regions in which the mutations occur, overlap with the translocation breakpoints. It is hypothesized that ASHM may thus favor chromosomal translocations and contribute to lymphomagenesis [93].

AID plays an essential role in aberrant targeting of the SHM. This was shown in AID transgenic mice that developed T-cell lymphomas with extensively mutated *C-MYC*, although chromosomal translocations were not detected in these mice. In contrast, in IL-6 transgenic mice that are prone to develop plasmacytomas AID appeared to be essential for the occurrence of IgV_H/*C-MYC* translocations [94]. Also, IL-4 and LPS-stimulated B-cells developed IgV_H/*C-MYC* translocations, but only in the presence of AID [95]. Finally, the vast majority of breaks and translocations involving the IgV_H locus are AID-dependent. In contrast to these reports, pristine-induced IgV_H/*C-MYC* translocations could occur in the absence of AID. Interestingly in this latter report outgrowth of translocation positive cells was dependent on the presence of AID[96]. Taken together, AID may induce aberrant SHM that can play a role in the occurrence of chromosomal translocations and thus may play a role in lymphomagenesis.

3. Outline of this thesis

Although the process of SHM is essential for the quality of the humoral immune response, it also puts the genomic integrity of the B cell in danger. It is the general assumption that this physiological process may play a role in lymphoma initiation but whether or not it is still active in certain subsets of B-NHL and thus plays a role in tumor progression has been debated for years. The discovery of AID has provided new means to investigate the activity of this DNA remodeling process. In **chapter two** we quantitatively measured the expression of this enzyme in an elaborate panel of primary B-NHL. In addition, we investigated if ongoing SHM may have played a role in lymphoma transformation. Detailed IgV_H gene analysis of these lymphomas enabled us to compared AID expression levels with the previously accepted manner to investigate ongoing SHM, i.e. intraclonal variation. In **chapter three** these topics were addressed for the specific group of B-CLL that has undergone transformation to a DLBCL (Richter's transformation). Furthermore, by structural analysis of the most variable region of the *Ig*, the CDR3 region, we investigated antigenic specificities of the BCR in this particular subgroup. In **chapter four** IgV_H gene and phenotypic analysis was performed on three unique cases of FL that showed a dedifferentiation towards acute lymphomblastic lymphoma. **Chapter five** describes four cases of FL of the gastro-intestinal tract. In order to learn more about their pathogenesis and their low tendency to disseminate outside the gastro-

intestinal tract we performed IgV_H mutation analysis and investigated the expression of α4β7, a well defined mucosal homing receptor. **Capter six** summarizes existing data about the somatic hypermutation process in FL initiation and progression and sheds some light on the role of the microenvironment, particularly the B-cell receptor ligands, in lymphomagenesis in mice and men. In **Chapter seven** we also investigated the effect of the microenvironment on tumor growth and apoptosis. We used a relatively new method to measure the expression of a large panel of apoptosis regulators and compared them between lymph node and peripheral blood samples of B-CLL patients. Finally **chapter eight** summarizes and discusses the results presented in this thesis.

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Expression of activation-induced cytidine deaminase is confined to B-cell non-Hodgkin's lymphomas of germinal-center phenotype.

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Expression of Activation-Induced Cytidine Deaminase is confined to B-non Hodgkin's lymphomas of germinal-center phenotype

2

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Abstract

Activation-induced cytidine deaminase (AID) is essential for somatic hypermutation and class switch recombination of the immunoglobulin (*IG*) genes in B cells. It has recently been proposed that AID, as the newly identified DNA mutator in man, may be instrumental in initiation and progression of B-cell non Hodgkin's lymphomas (B-NHL). We quantitatively measured, by real time RT-PCR, expression of AID and of the error-prone DNA polymerase ι (iota) in normal B cells and a comprehensive panel of B-NHL entities. In pre- and post-germinal center (GC) type B-NHLs, like in normal naive and memory cells, AID did not exceed background levels. However, half of Burkitt lymphomas tested were found to express AID, at most at levels comparable to those found in normal GC B cells. Thirty percent of diffuse large B-cell lymphomas also transcribed AID, some at supra-physiological levels. Of follicular lymphoma (FL) cases, only twenty five percent expressed significant amounts of AID. Moreover, within the group of GC-type B-NHLs, a statistically significant correlation between AID and Polι expression was found. By contrast, we observed no correlation between AID expression and mutation load neither with the degree of intraclonal diversity of *IG* variable heavy chain genes. Interestingly, in 2 of 7 follicular lymphomas with clinical and histological progression, selective outgrowth of AID-expressing clones occurred, suggestive for a role of the somatic diversification machinery in lymphoma transformation.

Introduction

To create the degree of antibody diversity necessary to combat the huge repertoire of microorganisms, immunoglobulin (*IG*) genes undergo several regulated alterations during B-cell development. The *IG* remodeling processes that occur during the germinal center (GC) stage of B-cell differentiation, albeit essential for the quality of the humoral immune response, are believed to bear an intrinsic risk of derailment [1]: Non-Hodgkin lymphomas (NHL) are in majority of B-cell origin and of GC or post-GC phenotype [2] and several *IG* gene-related chromosomal translocations of B-NHL entities now seem to result from mistakes in DNA diversification in the GC, in particular somatic hypermutation (SHM) and *IG* class switch recombination (CSR) [1]. It has also become clear that the longheld dogma that SHM is confined to the *IG* loci is incorrect and that other genes, like *BCL-6*[3] and *CD95*[4], can be substrates even in non-neoplastic B cells. Recently, Pasqualucci and colleagues [5] demonstrated that some reported translocation partners of *IG*, i.e. the proto-oncogenes *C-MYC, PIM-1, PAX-5,* and *TTF-1* are mutated in a proportion of diffuse large B-cell lymphomas (DLBCL), most likely due to an aberrantly targeted SHM machinery. Such promiscuous hypermutation activity is assumed to increase the chance that target genes become involved in chromosomal translocations and, perhaps more importantly, implies a new form of genetic instability.

SHM and CSR are now known to be highly related molecular processes both depending on the recently identified enzyme activation-induced cytidine deaminase (AID) [6]. Absence of *AID* in knockout mice [7] as well as in hyper-IgM syndrome type 2 patients [8] leads to absent CSR and defective SHM. Although AID shares homology with the RNA-editing enzyme APOBEC-1, recent reports strongly suggested that AID acts by direct deamination of cytidine residues in DNA [9] [10-12]. Most likely, SHM and CSR both start with the AID-triggered conversion of cytidine to uracil residues in the nontemplate DNA strand of the *IG* variable and heavy (H) chain switch regions respectively, with preference for certain hotspot motifs [12,13]. These mismatches can become fixed mutations if they are replicated without prior modification [14]. Alternatively, the created dU/dG pairs can be resolved by the base excision repair (BER)[9,15,16] and mismatch repair systems, involving uracil glycosylase, endonucleases and error-prone DNA polymerases. There is evidence that the polymerases Polι, Polη [17,18] and Polζ [19]are operational in these pathways. Of note, transgenic mice, with ectopic and deregulated AID expression, were found to die early due to development of epithelial and lymphoreticular neoplasms harboring several hypermutated non-IG genes[20]. This demonstrated that AID, as the first active genome mutator identified in man, has oncogenic potential.

Although it has been proposed that somatic gene diversification may be instrumental in the ongoing transformation process of B-NHLs, it is currently unknown to what extent the processes of SHM and CSR are indeed active in the group of B-NHLs. We previously noted that at least a proportion of FLs, in spite of their morphological and architectural resemblance

to normal GC B cells, and in spite of the somatically diversified nature of their *IG* genes, lack these typical GC functions [21-26]. With the knowledge recently obtained regarding the molecular basis of the *IG* gene remodeling processes this topic in B-NHLs can be studied in a more exact manner. We here present data on mRNA expression of AID and DNA polymerase ι in human tonsillar B cells and in an elaborate set of B-NHLs, covering most clinicopathological entities. Our findings demonstrate that AID expression closely correlates with the immunopathological phenotype of B-cell malignancies and that AID, as a genome mutator, may play a role in ongoing transformation of at least part of GC-stage derived B-NHLs.

Material and methods

Patient material

Tumor material was obtained from surgically removed lymph nodes that were snap-frozen in liquid nitrogen at the departments of Pathology of the Academic Medical Center in Amsterdam, the Westeinde Hospital in The Hague, the Academic Hospital in Groningen and the Spaarne hospital in Haarlem, The Netherlands. B-CLL samples were derived from peripheral blood obtained at the department of Hematology of the Academic Medical Center in Amsterdam. All lymphomas were classified according the WHO classification [2] criteria and judged at least by two expert pathologists. Of all Burkitt's lymphomas studied, a translocation involving *c-MYC* (t(8;14)(q24;q32) or t(8;22)(q24;q11)) was demonstrated by classical karyotyping and/or FISH [27]. To evaluate the relative number of tumor cells and to exclude the presence of residual GCs, extensive immunohistochemical analyses were performed. Thus it was ensured that in all B-NHL and B-CLL samples the relative contribution of tumor cells was at least 80%.

B cell purification and cell sorting

Tonsillar mononuclear cells were obtained by Ficoll density centrifugation (Pharmacia Biotech, Roosendaal, the Netherlands). B cells were purified by E-rozetting. Naive, GC and memory B cells were sorted by a FACS-Vantage (Beckton Dickinson, Erembodegem-Aalst, Belgium) based on double-stainings using a biotin-labeled mAb specific for CD38 (Caltag, Burlingame, CA), detected by Cy5-PE labeled streptavidine (Dako, Glostrup, Denmark), and a polyclonal FITC-labeled antibody specific for IgD (Dako) [28]. All samples analyzed contained the specific B-cell subpopulations at purity of at least 90%.

RNA isolation, cDNA synthesis and quantitative PCR analysis

Total RNA was isolated using the Trizol reagent (Life technologies, Breda, the Netherlands) and complementary DNA (cDNA) was synthesized using $pd(N)_6$ random primers

(Pharmacia) as described [29]. Quantitative real time PCR analysis was performed using a LightCycler (Roche, Almere, The Netherlands) [30]. AID was amplified using the primers 5'AGAGGCGTGACAGTGCTACA 3' and 5'TGTAGCGGAGGAAGAGCAAT 3' matching sequences in the 3' end of exon 2 and 5'end of exon 3, respectively. All reported AID splice variants are detected by this PCR (but not discriminated) except for the splice variant lacking the 3'end of exon 2 and whole exon 3 [31]. To adjust for variable cDNA input the relative levels of β-actin cDNA were determined using the primers 5'GGATGCAGAAGGAGATCACTG 3' and 5'CGATCCACACGGAGTACTTG 3'. Messages of the error-prone polymerases were amplified by quantitative RT-PCR using distinct forward and reverse primer combinations, i.e. 5' CATCAGGTTGTGGAGCAGTT 3' and 5' GGCAAACACCCATGAACTTT 3' for Pol β, 5' TGGTCGTGAGAGTCGTCAGT 3' and 5' ACCATTGGGGTCATCACATC 3' for Pol ι, 5' TGTGGGCAGATGATGCTAAG and 5' CCCGGTACTTGGTGAGGTTA 3' for Polη and 5' CGCGTCAGTTGGGACTTAAG 3' and 5' ACTATCGCCAACCTCAATGC 3' for Pol ζ, respectively. The FastStart DNA Master SYBR Green I reagent (Roche) was used for amplification with $MgCl_2$ at 4mM for AID, Pol β and Pol ζ, 3mM for β-actin and Polι, and 2.5mM for Pol η. After incubation at 95°C for 6 minutes, 40 cycles of amplification were performed, i.e. successively 10 seconds at 95°C, 5 seconds at 60°C and 5 seconds at 72°C. In each PCR run, a dilution series of one AID-expressing FL was used to construct a calibration curve. All PCR products were positively identified by melting curve analysis. Results are expressed as ratios of the relative values of AID and β-actin in comparison to the calibrator sample.

Variable heavy chain gene mutation analysis

IGVH genes of B-NHLs were amplified by RT-PCR, cloned and sequenced as described [29]. Of each lymphoma, 3 to 10 clones (on average 6 clones) were sequenced. The consensus *IGVH* sequence is divined as the nucleotide sequence that is shared by more than 50% of the clones. The intraclonal variation (ICV) was calculated as the mean number of nucleotide differences per clone compared to this consensus *IGVH* sequence.

Results and discussion

AID / β-actin mRNA ratios were measured in a variety of normal and malignant B-cell populations by real time RT-PCR using a LightCycler. In normal naive as well as memory B cells, originating from peripheral blood and tonsil, no significant AID was amplified yielding AID / β-actin ratios below 0.8 (Fig.1 and data not shown). In accordance with previous reports [6,32,33], significant AID expression was found in sorted GC B cells by our quantitative PCR approach yielding relative values of 1.5, 2.8 and 6.3
(with an average of 3.5).

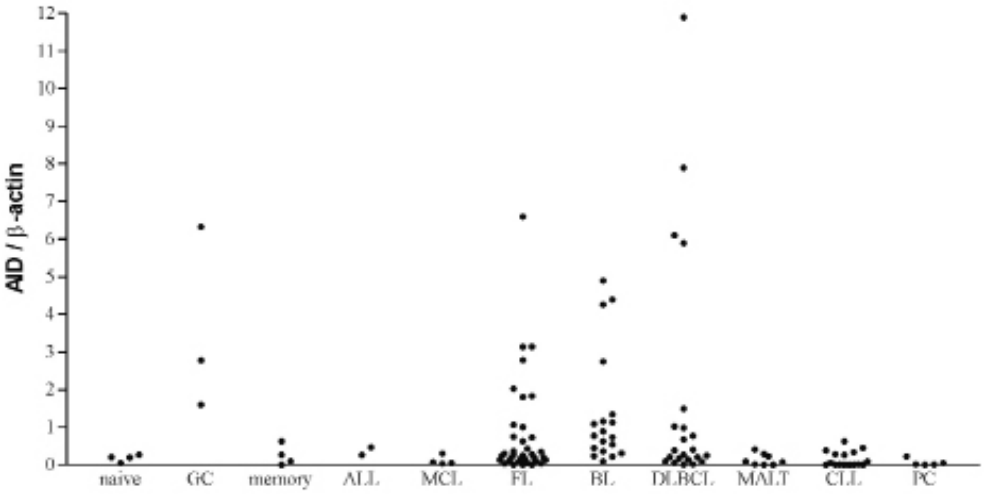

Figure 1. AID expression in normal B cell populations and B-NHLs. Quantitative RT-PCR analysis of AID and β-actin was performed on naive, GC and memory B cell populations and various primary B-NHLs. Each dot represents the average value of at least three AID / β-actin ratio measurements. The inter-experimental variation was 30% on average.

We subsequently investigated an extensive panel of B-NHLs representative for all maturational stages, i.e. 2 precursor B-cell lymphoblastic leukemias (B-ALL), 4 mantle cell lymphomas (MCL), 36 follicular lymphomas (FL), 22 DLBCL, 18 Burkitt's lymphomas (BL), 9 low-grade extranodal marginal zone B-cell lymphomas of mucosa-associated lymphoid tissue (MALT lymphomas), 18 chronic lymphocytic leukemias (B-CLL) and 5 plasmacytomas (PC) (Fig.1). In remarkable accordance with their phenotypes, in B-NHLs of pre-GC phenotype, i.e. B-ALL and MCLs, and of post-GC phenotype, i.e. MALT lymphomas, B-CLL and plasmacytomas, we measured AID / β-actin ratios that did not exceed those found in normal naive or memory B cells. Of note, among the 15 B-CLLs, 9 cases expressed *IGVH* genes with mutation frequencies above 2%. Moreover, also after B-cell antigen receptor-mediated stimulation *in vitro* of these mutated and unmutated B-CLL variants, no AID expression was induced up to quantifiable levels (not shown). Our findings seem to contradict recent papers reporting on AID expression in B-CLL [31-33]. In most of these studies however, AID mRNA expression was measured by non-quantitative RT-PCRs and by consequence the actual expression levels are difficult to judge. Recently, McCarthy and colleagues [32] reported on high AID expression in a proportion of, mostly unmutated, B-CLL. The authors however show, by quantitative AID mRNA measurement using TaqMan technology, that the expression levels found in the B-CLL were in all cases less than 5% of those found in GC cells. This thus is concordant with our measurements albeit in our RT-PCR assay such levels are not quantifiable. We therefore think that the mutations found in a proportion of B-CLL are unlikely to result from an active or inducible mutation apparatus but are introduced prior

to or at early stages of cellular transformation.

Strikingly, AID was only expressed at significant levels in lymphomas with a GC phenotype: in 9 of the 18 BLs AID was expressed**,** at most at levels comparable to those found in normal GC B cells. This is in agreement with evidence that in BLs [34] and in BL cell lines[35,36] the SHM / CSR machinery is active or can be induced. Of 22 DLBCLs tested, 7 (32 %) expressed measurable AID mRNA, 2 of which (overall 9 %) at relatively high levels. This is an intriguing finding since artificial over-expression of AID is known to confer mutation of non-*IG* genes, also in T cells, fibroblasts [37-39]and even bacteria [9]. Among the panel of 36 FLs, only 9 (25 %) expressed AID above detection level. These AID-expressing FLs were, as a group, not obviously distinctive with respect to histology, immunophenotype nor stage.

We quantitatively measured in the panels of B-NHLs, mRNA expression of the DNA polymerase Polβ and members of the Y family of error-prone DNA polymerases, i.e. Polι, Polη and Polζ. Interestingly, among the AID expressing B-NHLs, a statistically significant correlation between the expression levels of AID and Polι, (Spearman rank correlation coefficient ρ of 0.6, $p<0.002$) (Fig. 2), but with none of the other polymerases (not shown), was observed. When the AID positive B-NHLs were separated according to subtype, a statistically significant correlation between AID and Polι was observed within the group of BLs ($\rho=0.62$, $p<0.05$). The values calculated for the both FLs and DLBCLs did not reach statistical significance. Still it is remarkable that in the subgroup of DLBCL, the 3 cases with the highest Polι expression levels also displayed the highest mRNA levels for AID. The trend that we observed within our panel of B-NHLs, seems compatible with recent RNAi-based gene inactivation studies showing that SHM depends on Polι [40] and suggests co-regulation of expression of two key enzymes of the somatic gene diversification process in B-NHLs

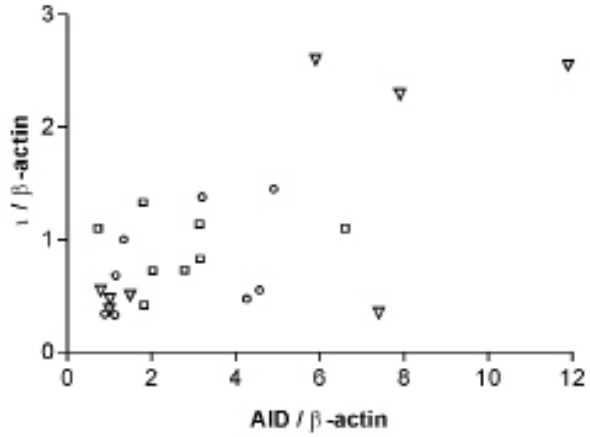

Figure 2. Relation between AID and polι mRNA expression in the group of GC-derived B-NHL entities. Correlation between AID / β-actin and polι / β-actin ratios in AID expressing FLs (squares), BLs (rounds) and DLBCLs (triangles).

The finding that a minority of FLs express AID is in support of our previous proposal that, in contrast to the general belief, at least a part of these B-NHLs have not retained GC-specific functions like SHM and CSR [21,22,29]. To substantiate this notion, we compared of individual FLs, BLs and DLBCLs the measured AID levels with the total number of consensus mutations and the degree of intraclonal sequence variation (ICV) in their *IGVH* genes, as far as these could be assessed (Fig. 3).

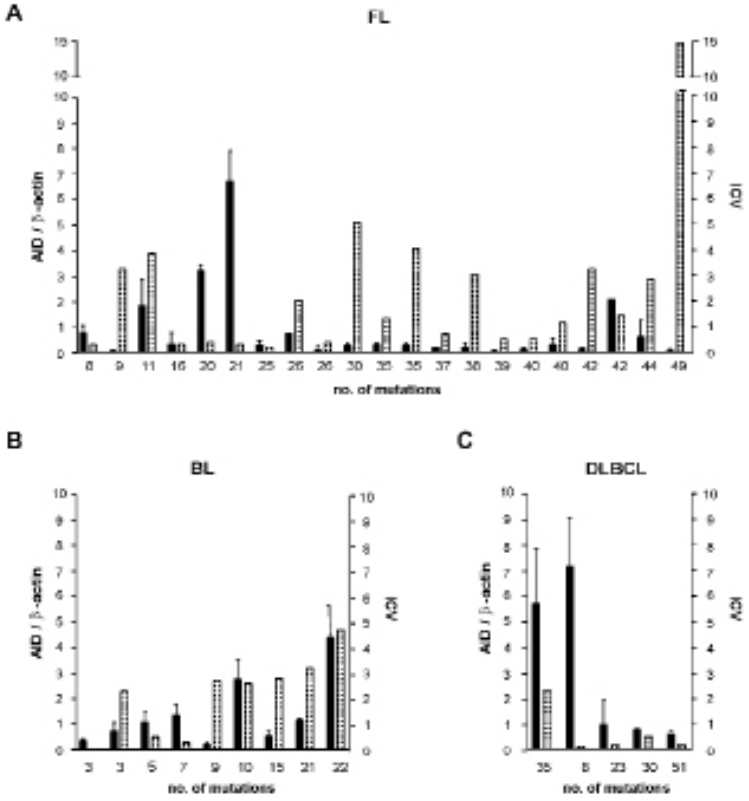

Figure 3. AID expression compared with intraclonal *IGVH* gene diversity as measured in individual B-NHLs. AID / β-actin ratios with the values of intraclonal *IGVH* gene sequence variation (mean number of nucleotide differences observed per clone compared to the consensus sequence) as determined in individual FLs (a), BLs (b) and DLBCLs (c). On the x-axis are plotted the total number of consensus mutations present in the different B-NHLs.

It was striking that for all three B-NHL types, this comparison revealed no obvious correlation between either of these three parameters. With respect to the group of FLs (Fig. 3a), different categories could be discerned: FLs lacking ICV, with or without significant AID expression. Next, FLs with significant ICV but no detectable AID expression. This subgroup is of importance as it proves our notion that ICV is not necessarily a reflection of active SHM during the tumor stage but rather a remnant of earlier stages of transformation. By consequence,

the mutation patterns found in these FLs, which resemble those of normal antigen-selected B cells, neither result from continued antigen-receptor based selection processes but more likely are left over from the initial GC reactions in which the respective progenitor tumor cells had been engaged. Of note, this subgroup also included the previously reported FL8, which harbored both IgM- and IgG-expressing tumor cells of the same clonal origin (with 30 and 35 consensus mutations respectively) [21,22,29]. In spite of the abundance of H chain isotype switch variants among the neoplastic cells, we have not been able to detect CSR excision circles in the tumor tissue [22]. Moreover, in this FL the degree of ICV had decreased instead of increased over a nine year interval [21,22]. Our interpretation that in this FL, and most likely also other FLs, neither CSR nor SHM proceeds is again corroborated by the finding of absent AID expression. In two FLs (with respectively 11 and 42 consensus mutations), significant ICV as well as AID expression was detected. A similar pattern was obtained for BLs (Fig. 3b), with cases showing significant ICV without quantifiable AID levels (with 3, 9 and 15 consensus mutations, respectively) and vice versa (with 5 and 7 consensus mutations, respectively). One BL, with 3 consensus mutations, lacked AID and ICV, whereas in 3 BLs (with 10, 21 and 22 consensus mutations respectively) both AID expression and ICV of the *IGVH* genes was found. In these latter cases the somatic diversification machinery is thus potentially active.

For reasons not determined, we managed to resolve the *IG* gene configuration only of a limited number of DLBCLs (Fig. 3c). Although this may thus well provide a biased view, it is apparent that AID is not preferentially expressed in cases with the highest number of consensus mutations; two out of 5 DLBCLs (with 35 and 8 consensus mutations, respectively) harbored significant AID mRNA levels. Interestingly, the first case also displayed ICV of the *IGVH* gene and was one of the three DLBCLs with the highest levels of Pol ι expression (Fig.2). By contrast, the second AID^+ DLBCL, with 8 consensus mutations, lacked ICV and Polι expression. We conclude that the hypermutation machinery is likely to be active in at most a minority of the FLs and DLBCLs. In BLs the relative number with ongoing gene diversification may be higher. Furthermore, knowing that AID expression is mandatory for the hypermutation process, the current findings confirm our previous notion that ICV alone is an inappropriate parameter to read out hypermutation activity in B-NHLs. Finally, we measured AID mRNA in 7 FLs with clinical progression over time (Fig.4). In 6 of these lymphomas (FL3, FL6, FL24, FL66, FL83 and FL91), progression was also evident histologically. In FL24, AID expression was measurable at both time-points (FL24-'95 and FL24-'99) with a slight increase over time. Interestingly, in 2 cases significant AID expression was only detected at the second time point (FL3-'95 and FL6-'98). The fact that in these 2 cases AID was not expressed at presentation (FL3-'93 and FL6-'94, respectively) indicates selective outgrowth of subclones with AID transcription and thus potentially with an active mutation machinery. This subgroup among the FLs is of interest as it may harbor

a clue for the questions whether AID plays a role in the process of tumor progression and, if so, at what stage the mutations found in the proto-oncogenes in DLBCLs, as described by Pasqualucci and collegues [5], are being introduced. In conclusion, AID is expressed in a proportion of B-NHLs of GC phenotype only. The finding of DLBCL with abnormally high AID levels as well the observed selective outgrowth of AID-expressing tumor cells in FL suggest that SHM-related genetic instability is not only essential in tumor initiation but may also be instrumental in ongoing transformation of GC derived B-cell malignancies. It is envisaged that if lymphomas with a "mutator" phenotype are more prone to progress to higher-grade malignancies, assessment of SHM activity of low-grade B-NHLs will become of clinical relevance.

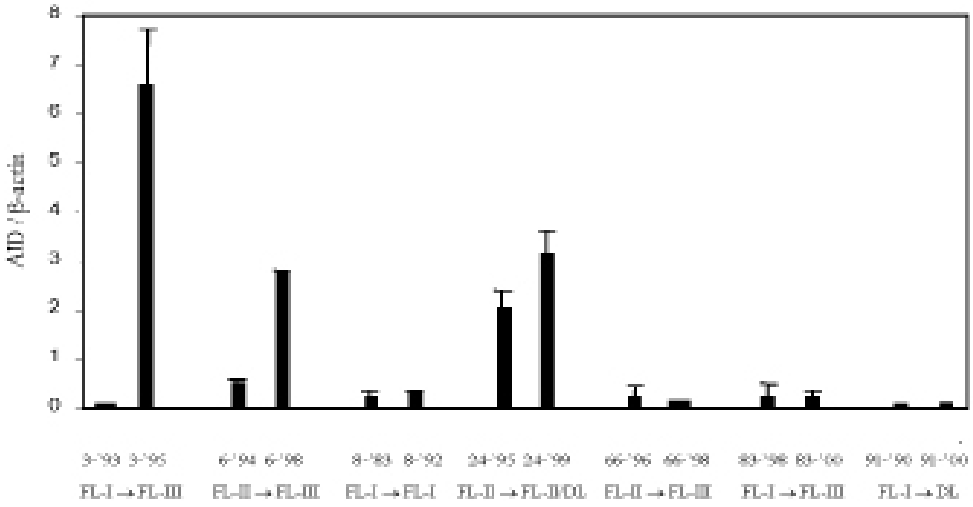

Figure 4. AID expression in FLs with progression. AID / β-actin ratios at successive time-points of 7 FLs that showed clinical progression. Histological grading, according to the WHO classification [2], is denoted below the time-points of each lymphoma. FL-II / DL below FL24-'99 signifies a partially follicular, partially diffuse growth pattern. The increase in AID expression as observed in three FLs was statistically significant in FL3 and FL6.

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Antigen receptors and somatic hypermutation in B-cell Chronic Lymphocytic Leukemia with Richter's transformation

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Antigen receptors and somatic hypermutation in B-cell Chronic Lymphocytic Leukemia with Richter's transformation

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Abstract

Background and objective: Activation-induced cytidine deaminase is essential for somatic hypermutation and class switch recombination of the immunoglobulin genes in B cells. It has been proposed that aberrant targeting of the somatic hypermutation machinery is instrumental in initiation and progression of B-cell non Hodgkin's lymphomas. In this study, we investigated the B-cell receptor and the role of the somatic hypermutation machinery in B-cell chronic lymphocytic leukemias (B-CLL) prior and after transformation to a lymphoma of a higher malignancy grade (Richter's transformation).

Design and Methods: We investigated the activity of the somatic hypermutation machinery in 9 B-CLL and secondary diffuse large B-cell lymphomas by measuring the expression of Activation-induced cytidine deaminase, in combination with mutation analysis of immunoglobulin (Ig) and non-Ig genes. Furthermore, the structure of the antigen receptors of B-CLL known to have developed a Richter's syndrome (RS B-CLL) was analyzed by comparing the most variable region of the Ig, the CDR3 region, to CDR3 sequences present on GenBank.

Results and Interpretation: Ig variable heavy chain (IgV_H) gene studies revealed that Richter's transformation occurs almost exclusively in unmutated B-CLL. Furthermore, AID expression and somatic hypermutation activity of most RS B-CLL were found higher than those of control (non-transforming) B-CLL. Finally, comparison of the IgV_H-CDR3 regions showed a remarkable amino acid sequence homology between 2 RS B-CLL of our panel and 2 RS B-CLL described in literature.

Conclusion: The combined findings suggest a role for the Ig gene diversification apparatus during Richter's transformation and show that distinct RS-B-CLL may recognize recurrent antigenic epitopes.

Introduction

Richter's syndrome (RS) is the rare occurrence of a histologically and clinically aggressive secondary lymphoid malignancy in a patient with B-cell chronic lymphocytic leukemia (B-CLL) (1, 2). In approximately 3-5 % of B-CLL cases, a lymphoma of a higher malignancy grade develops, reducing the mean disease-free survival to 6 months (2). Usually the high-grade lymphoma is classified as a diffuse large B cell lymphoma (DLBCL) and less commonly as a Hodgkin's lymphoma (3).

B-CLL is characterized by an accumulation of long-lived, monoclonal $CD5^+$ $CD23^+$ mature B cells that express low levels of membrane-bound immunoglobulin (1). About 50% of the B-CLL harbor somatic mutations in their immunoglobulin variable heavy chain (IgV_H) genes (4). Although the difference in mutation status suggests a different cell of origin, gene expression profiling revealed that mutated and unmutated B-CLL are both most similar to normal memory B cells (5, 6). B-CLL express an IgV_H gene repertoire clearly distinct from the IgV_H repertoire of normal B cells of any lineage or maturational stage (4). It has been reported by others and us that ~ 19 % of B-CLL, mostly unmutated, express IgV_H-CDR3 amino acid sequences homologous with CDR3 regions of other B-CLL (so called inter-B-CLL CDR3 homology) (7-11). As yet, at least 8 B-CLL IgV_H-CDR3 homology groups have been defined (10). The occurrence of highly homologous B-cell receptors among B-CLL strongly suggests that they recognize a limited set of distinct antigenic determinants.

It is unknown to what extent the somatic hypermutation machinery is active in B-CLL. It is generally assumed that B-CLL have a low tendency to acquire additional mutations over time (12). Accordingly, the overall expression level of the enzyme that is essentially required for both somatic hypermutation and class switch recombination, i.e. Activation-induced cytidine deaminase (AID) (13, 14), is very low in blood-derived B-CLL samples as compared to those of purified germinal centre (GC) B cells (15, 16). It has been reported that only a small fraction (<1%) of circulating B-CLL cells, particularly of the IgV_H-unmutated subgroup, expresses AID (17). However, in whole lymph node samples, where the CD40-expressing B-CLL cells are in close contact with CD40L-expressing $CD4^+$ T cells, overall AID expression was found to be higher (18). In accordance, *in vitro* stimulation of B-CLL cells by $CD4^+$ T cells and anti-B-cell receptor antibodies, induces somatic hypermutation in the IgV_H genes (19).

It has been proposed that promiscuous targeting of the somatic hypermutation machinery may be an initial event in the development of a number of DLBCL (20). It is not known whether this mechanism also applies to progression of low-grade B-non Hodgkin's lymphomas (B-NHL). In the current study, we analyzed the B-cell receptor and the process of somatic hypermutation in a panel of B-CLL with documented transformation. Our data suggest restricted B-cell receptor specificities and an active somatic hypermutation machinery in B-CLL undergoing Richter's transformation.

Material and Methods

Patient material.

All lymphomas were diagnosed according to the WHO classification system (1). Lymph node material of *RS1*, *RS8* and the control B-CLL was freshly frozen in liquid nitrogen directly after surgical removal. Immunohistochemical analysis of *RS1* and *RS8* revealed that more than 80% of the tissue consisted of tumor cells. Of all other RS cases and of the peripheral blood samples of the control B-CLL, cell suspensions were frozen in 20% DMSO (Merck, Darmstadt, Germany) in FCS (Invitrogen, Breda, The Netherlands). This study was conducted in accordance with the ethical standards in our institutional medical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, revised in 1983.

FACS analysis and cell sorting.

The following monoclonal antibodies (mAb) were used for FACS analysis: PE-conjugated anti-CD23 (clone EBVCS-5; Dako, Glostrup, Denmark), PE-conjugated or FITC-conjugated anti-CD5 (clone L17F12; Dako) and APC-conjugated or PerCPCy5.5-conjugated anti-CD19 (clone SJ25C1; Becton Dickinson Biosciences, Erembodegem-Aalst, Belgium). The following polyclonal antibodies were used: FITC-conjugated anti-Igκ, anti-IgM, anti- IgD and anti-IgA, PE-conjugated anti-Igλ, and anti-IgG (polyclonals from Southern Biotechnology Associates, Birmingham, AL). FACS analyses revealed that the cell suspensions of *RS3*, *RS9*, *RS10, RS11c* and *RS12* consisted of >90% tumor cells, whereas the peripheral blood sample of *RS11a* consisted of 60% tumor cells. Of *RS4* and *RS6*, the $CD5^+$, $CD19^+$ tumor cells were isolated using a FACS-Aria (BD Biosciences) cell sorter what resulted in more than 97% pure tumor samples. Germinal centre (GC) B cells were sorted as described previously (15, 21).

Immunohistochemistry.

AID was visualized in formalin-fixed, paraffin-embedded tissue sections using a rat monoclonal antibody (22). After deparaffination, blocking and antigen retrieval the slides were incubated overnight at 4°C with the primary antibody (1:1000), followed by application of an HRP-conjugated rabbit-anti-rat antibody (1:200, Dako). Subsequently, biotin-free tyramide signal amplification (Dako CSAII kit) enabled detection of AID, which was visualized with Nova Red (Vector). A hyperplastic tonsil functioned as a positive control, omission of the primary antibody as a negative control. Monoclonal antibodies specific for CD5 (Lab vision, Neomarkers, Fremont, CA), CD23, BCL6, and Ki67 (all from Dako) were used. Antibody detection was performed with the Powervision$^+$ system (ImmunoVision Technologies, Daly

City, CA) and succeeded by peroxidase visualization with 3,3'-diaminobenzidine (DAB) (Sigma), 0.03% H_2O_2 in Tris-HCl pH 7.6.

DNA isolation, RNA isolation and cDNA synthesis.

RNA and DNA were isolated using the Trizol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's description. First-strand complementary DNA (cDNA) was synthesized as described previously albeit 5'-$(dT)_{14}$-d(A/G/C)-d(A/G/C/T)-3' primers were used (15).

Amplification, cloning and sequencing.

IgV_H transcripts were amplified using a mixture of forward primers located in the FR1 regions of the *IgV_H* gene families V_H1 to V_H6 or alternatively in the FR3 region of V_H1 to V_H6 (23) in combination with one of the FAM-labeled reverse primers located in Cμ, Cδ, Cα or Cγ regions (24). The PCR reaction was performed as described previously using a 30 cycles program (24) and run on an ABI PRISM 3100 automated sequencer in the presence of either 1pM ROX 500 or 1pM ROX1000 marker (Applied biosystems, Warrington, UK). Results were analyzed using the program Genescan analysis (Applied Biosystems). When a monoclonal tumor population was present, IgV_H amplicons were cloned into the pTOPO-TA vectors and transformed into TOP10 bacteria according to the manufacturer's description (Invitrogen) and 8 to 16 clones were sequenced of each lymphoma. Sequencing on both strands was performed by an ABI PRISM 3100 automated sequencer (Applied Biosystems) using the big dye-terminator cycle-sequencing kit (Perkin Elmer Corporation). The consensus *IgV_H* sequence is defined as the nucleotide sequence that is shared by more than 50% of the clones. Nucleotide alterations that are present in less than 50% of the clones are considered as intraclonal variation. Of note, according to our nomenclature, nucleotide alterations that are present in multiple clones (confirmed nucleotide differences) but are present in <50% of the clones are thus still regarded as intraclonal variation. The amount of intraclonal variation (ICV) was calculated as the mean number of nucleotide differences per clone compared to the consensus *IgV_H* sequence and was considered significant when it was higher than the Taq error rate. To determine the Platinum *Taq* error rate of our experimental design, 48 clones of HPRT were sequenced using the primers 5'TTCCTCCTCCTGAGCAGTCAGC3' and 5'GCGATGTCAATAGGACTCCAGATG3'. These clones were generated according to the same PCR and cloning procedures as used for the *IgV_H* genes. The Taq error frequency thus established is 0.2 per 300 bp. *BCL6* was amplified using the primers 5'CCGCTGCTCATGATCATT3' and 5'CAGACTCGAGTCTTCCCATGGATCCACC3'. *PIM1* was amplified and sequenced using the primers 5'AGCAGCAGCAGCAACCACTAG3' and 5'CTCTCCCCAGTCGGAAATCC3'. The PCR mixtures contained 1x *pfx* amplification

buffer, 1U platinum *pfx* DNA polymerase (Invitrogen), 1mM (BCL6) or 2.5mM (PIM1) $MgSO_4$, 0.2mM of each dNTP, 0.5mM of each primer and 1x enhancer solution.
Both BCL6 and PIM1 PCR reactions started with 3 minutes at 94°C, followed by 39 cycles of 30 seconds at 94°C, 1 minute at 55°C and 1 minute at 68°C. The reaction was terminated for 4 minutes at 68°C. BCL6 and PIM1 amplicons were cloned as described above and 12-24 clones were sequenced. BCL6 was sequenced using the primers 5'CCGCTGCTCATGATCATT3' in combination with 5'GCAAGCGAGAAAAGAGGAA3' and 5'GTACGGCTTGTGATCTCTCT3' in combination with 5'CAGACTCGAGTCTTCCCATGGATCCACC3'. *BCL6* and *PIM1* were amplified from DNA. Since DNA harbors two alleles of all genes, the consensus *BCL6* and *PIM1* sequence was defined as the nucleotide sequence that is shared by more than 25% of the clones.

QRT-PCR reactions.
Quantitative RT-PCR analyses were performed using a LightCycler (Roche, Almere, The Netherlands). AID was amplified using the primers 5'AGAGGCGTGACAGTGCTACA 3' and 5'TGTAGCGGAGGAAGAGCAAT 3' matching sequences in the 3' end of exon 2 and 5'end of exon 3, respectively. All reported AID splice variants are detected by this PCR (but not discriminated) except for the splice variant lacking the 3'end of exon 2 and whole exon 3 en 4 (25). β-Actin was amplified using the primers 5'GGATGCAGAAGGAGATCACTG 3' and 5'CGATCCACACGGAGTACTTG 3'. The PCR reactions for both AID and β-actin were performed in a volume of 10 µl containing 2µl cDNA, 1 µl FastStart DNA Master ^{PLUS} SYBR Green I mix (Roche) and 0.5 pM forward and reverse primers. The PCR protocols to amplify AID and β-actin started with 95°C for 6 minutes, after which 40 cycles of amplification were performed, i.e. successively 10 seconds at 95°C, 5 seconds at 60°C (AID) or 61°C (β-actin) and 5 seconds at 72°C (AID) or 8 seconds at 72°C (β-actin). Melting curve analysis was performed to check for PCR specificity. Starting concentrations of mRNAs and PCR efficiencies for each sample were calculated using the LinRegPCR computer program as described before (26). Results are expressed as ratios of the calculated values of AID and β-actin.

In vitro stimulation of B-CLL cells.
B-CLL cells were cultured for four days in 24-wells plates (Costar, Corning NY, USA). Each well contained 2 x 10^5 B-CLL cells and 1 x 10^5 L cells as a control or 1 x 10^5 CD40L-transfected L cells and 400 U/ml IL4 (Strattmann, Hannover, Germany) with and without anti-IgM (clone MH15/1)(Sanquin, Amsterdam, The Netherlands) coupled CNBR-activated sepharose beads (Amersham biosciences, Uppsala, Sweden). As a positive control peripheral blood B cells of healthy volunteers were stimulated with each experiment.

Results

B-CLL with Richter's transformation.

Tumor samples of 9 B-CLL that underwent clinical and histological progression to a DLBCL were analyzed. *RS1*, *RS3*, *RS4 RS6* and *RS10* presented as a monoclonal population of small $CD5^+$, $CD19/CD20^+$, sIg^{low} B-CLL cells which over time transformed into a DLBCL (Table 1). *RS8* already showed signs of transformation at presentation with, next to small tumor cells, a subpopulation of centroblast-like cells with abundant basophilic cytoplasm and irregular nucleoli. In a lymph node sample of *RS8* one year later, the percentage of centroblast-like cells had clearly increased. *RS9*, *RS11* and *RS12* have been described previously as *case 9*, *case 3* and *case 8* respectively (27).

Table 1. 9 B-CLL with clinical and histological progression to DLBCL.

RS	V_H	D	J_H	CDR3 (no. amino acids)
RS1	V_H1-69 (V_H1.2)	3-10 (fr.1)	J_H6b	GGRQELLWFGEFDYYYYGMDV (21)
RS3	V_H4-4b (V_H4.22)	5-12 (fr.1)	J_H4b	GLNIVATGDY (10)
RS4a	V_H4-39 (DP79)	6-13 (fr.1)	J_H5b	NSGYSSSWFRGYSWFDP (17)
RS4b	V_H4-39 (DP79)	6-13 (fr.1)	J_H5b	NSGYTSSWFRGYNWFDP (17)
RS6	V_H5-51 (DP73)	n.a.	JH2	RPLQWPLERYWYFDL (15)
RS8	V_H3-30/30.5(DP49)	3-22 (fr.2)	J_H6c	GGDYYDSSGYGLYYYYYYYYMDV (22)
RS9	V_H1-69 (DP10)	2-21 (fr.2)	J_H4b	VAGVAYCGGDCYWREYYFDY (20)
RS10	V_H3-74(DA8)	3-16 (fr.2)	J_H3b	DAWRPARPAYYDYV (14)
RS11	V_H3-11 (DP35)	3-09 (fr.2)	J_H5b	DSVWYYDILTGYSPQLVSYNWFDP (24)
RS12	Vv1-8 (DP15)	n.a.	J_H2	ASSYDSGDYYYSLCLL (16)

BM indicates bone marrow; LN, lymph node; nd not done; PB, peripheral blood. * Time interval in months between samples ‡ RS9, RS11 and RS12 are previously described as Case 9, Case 3 and Case 8 (27)

IgV_H genes and CDR3 regions of B-CLL with Richter's transformation.

To establish the clonal relationship between the tumor populations at presentation and after relapse, the rearranged *Ig* V_HDJ_H genes were amplified by RT-PCR and sequenced (Table 2). In all RS cases, the B-CLL and DLBCL cells proved clonally related. Interestingly, sequence analysis revealed that the IgV_H genes of all 9 RS cases in our panel were unmutated (< 2% consensus mutations) (Table 4). The consensus *Ig* V_HDJ_H sequences of most of the RS cases remained unaltered over time. However, *RS4* and *RS6* had each acquired an additional consensus mutation (i.e. a mutation found in more that 50% of the molecular clones) in

their respective IgV_H genes after transformation (Table 4). *RS4* also acquired an additional replacement mutation in the third complementary determining region (CDR3) after transformation in more than 50% of the clones (Table 2).

Table 2. Immunoglobulin variable heavy chain genes and CDR3 amino acid sequences of 9 B-CLL with clinical and histological progression. CDR indicates complementarity determining region; n.a., The D gene was not assigned.

RS	time*	diagnose	Source	CD5	CD23	Ig class
RS1a		B-CLL	nasopharynx	+	+	IgM, IgD
RS1b	20	DLBCL	nasopharynx	+	+	IgM, IgD
RS3a		B-CLL	LN	nd	nd	IgM, IgD
RS3b	5	DLBCL	LN	nd	-	IgM, IgD
RS4a		B-CLL	BM	+	+	IgG
RS4b	14	B-CLL/DLBCL	PB	+	nd	IgG
RS6a		B-CLL	LN	+	+	IgM, IgD
RS6b	86	DLBCL	LN	+	+	IgM, IgD
RS8a		B-CLL/DLBCL	LN	+	+	IgG
RS8b	4	B-CLL/DLBCL	LN	+	-	IgG
RS9a‡		B-CLL	PB	+	+	IgM, IgD
RS9b‡	2	DLBCL	colon	+	+	IgM, IgD
RS10a		B-CLL	PB	+	+	IgM, IgD
RS10b	10	DLBCL	PB	+	+	IgM, IgD
RS11a‡		B-CLL	PB	+	+	IgM, IgD
RS11c‡	13	DLBCL	BM	+	+	IgM, IgD
RS12a‡		B-CLL	PB	nd	nd	IgD
RS12b‡	50	DLBCL	PB	+	+	IgD

The IgV_H-CDR3 region is the most hypervariable region of the Ig and is considered to contribute most to its antigenic specificity. Nevertheless, ~ 19% of B-CLL, mostly unmutated, express CDR3 sequences with homology to CDR3s of other B-CLL (inter-B-CLL CDR3 homology), which suggests that a limited set of distinct antigenic determinants is recognized by these B-CLL. This prompted us to investigate the IgV_H-CDR3 region of B-CLL known to have developed a Richter's syndrome (henceforth called RS B-CLL). The IgV_H-CDR3 amino acid sequences of the RS B-CLL described in this study and all RS B-CLL described in literature were compared to all CDR3 amino acid sequences available on GenBank (Table 3). For this purpose, we used the NCBI Protein-Blast program with the option "search for short nearly exact matches"(BLASTP2.2.6[apr-09-2003]) as reported previously (10).

Briefly, CDR3 regions consisting of at least 7 amino acids were analysed. An IgV_H-CDR3 sequence was considered to be homologous to other CDR3 sequences (i) if sharing at least 75 % amino acid sequence homology. (ii) A length difference between the CDR3 sequences was allowed if not exceeding 3 amino acids (maximum gap of 3 amino acids).

Table 3. Homology between IgV_H-CDR3 amino acid sequences of the 8 RS B-CLL and IgV_H-CDR3 amino acid sequences present on GenBank.

RS	Reference	CDR3 homology	Patient/Clone †
RS1	this study	1 normal B cell clone	ya0208
RS4a	this study	1 RS	CLL57
		3 B-CLL	CLL8, CLL9 and CLL202
		1 normal B cell clone	1HI81
RS4b	this study	1 RS	CLL57
		4 B-CLL	CLL8, CLL114, CLL202, CLL209
		1 normal B cell clone	1HI81
RS8	this study	1 RS	case 3
		1 B-CLL	CLL32
		1 anti-polysacc. of N. Meningitis Ab	SC15
case 3	Matolcsy et al.(29)	1 RS	RS8 (this study)
		1 EBV B cell in AITL	case2
		1 anti-polysacc. of N. Meningitis Ab	SC15
		1 anti-natural Sm Ab	BUD94
		1 anti-Rota virus Ab	RVI-22
		2 normal B cell clones	102-17 and MBT-159
B-CLL57	Ghiotto et al.(11)	1 RS	RS4a/b (this study)
		6 B-CLL	CLL39, CLL114, CLL209 CLL7, CLL8 and CLL9
		2 normal B cell clones	1HI81 and SC77U-44
RS 3557	Matolcsy et al.(30)	2 normal B cell clones	2CB4N2, A29A29
B-CLL4	Aoki et al.(39)	1 B-CLL	YarVH*
case 2	Ohno et al.(38)	1 normal B cell clone	PBT-16
		1 anti-Staphylococcal protein A Ab	4D5

Ab indicates antibody; AITL, angioimmunoblastic T cell lymphoma.

* B-CLL YarVH was 73% homolgous to B-CLL4 instead of at least 75% like the other cases in this table.

† GenBank accession numbers: ya0208, AB067329; CLL57, X84339; CLL8, AY486198; CLL9, AY486207; CLL202, AY268373; 1HI81, Y09249; CLL114, AY268372; CLL209, AY300037; CLL32, AY486216; SC15, AF115134; BUD94, Z46379; RVI-22, AY686908; 102-17, AF028108; MBT-159, U32960; CLL39**,** X84336**;** CLL7, AY486206; SC77U-44, AF174118; 2CB4N2, AY671324; A29A29, AF460484; YarVH, AF099199; PBT-16, U3220; 4D5, PH1650.

Table 4. Mutations and intraclonal variation of IgV_H, BCL6 and PIM1 in 5 RS B-CLL and 15 control B-CLL.

RS		IgV_H		BCL6		PIM1	
	AID/β-actin x 10^{-04}	mut*	ICV†	mut‡	ICV†	mut‡	ICV†
RS1a	36.1	0	≤ 0.2 (12)	1	0.4 (12)	0#	≤ 0.2 (16)
RS1b	nd	0	≤ 0.2 (11)	2	0.4 (7)	0#	0 (6)
RS3a	0.9	2	≤ 0.2 (9)	0	0.5 (23)	nd	nd
RS3b	0.9	2	≤ 0.2 (16)	0	0.5 (12)	nd	nd
RS4a	10.1	0	0.3 (12)	0¶	0.3 (17)	nd	nd
RS4b	140.0	1	≤ 0.2 (23)	0¶	≤ 0.2 (16)	nd	nd
RS6a	nd	0	≤ 0.2 (12)	nd	nd	nd	nd
RS6b	50.0	1	0.3 (12)	nd	nd	nd	nd
RS8a	8.3	0	≤ 0.2 (23)	0¶	≤ 0.2	0#	≤ 0.2 (7)
RS8b	5.0	0	≤ 0.2 (23)	0¶	≤ 0.2	0#	≤ 0.2 (9)
CLL M§	0.7	17	na	na	na	na	na
CLL UM“	0.2	0	na	na	na	na	na

CLL M indicates CLL cases with >2% IgV_H mutations; CLL UM, <2% IgV_H mutations; ICV, intraclonal variation; mut., mutation; nd, not determined. *IgV_H mutations are defined as nucleotide differences present in more than 50% of the clones, as compared to the germline sequence. †The intraclonal variation is indicated as the mean number of nucleotide differences observed per ~300 nucleotides per clone. In parentheses the number of clones that were sequenced. ‡BCL6 and PIM1 mutations are defined as nucleotide differences that were present in ≥25 % of the sequenced clones, as compared to the BCL6 and PIM1 sequences published on genbank (AY189709 and AF386792 respectively). §Values are the average of 9 mutated B-CLL “Values are the average of 6 unmutated B-CLL
¶A polymorphism was found in BCL6 at position 754 #A polymorphism was found in PIM1 at position 1039.

Of the 18 RS B-CLL that were thus studied, 8 (44 %) fulfilled our criteria for CDR3 homology with CDR3 amino acid sequences present on GenBank (Table 3). The CDR3 regions of these 8 RS cases were homologous to the CDR3 regions of 12 normal B-cell clones and 9 B-CLL without reported transformation (Table 3). These latter B-CLL all expressed unmutated IgV_H genes, except B-CLL *YarVH* (28) whose IgV_H genes harbored 5 mutations. Interestingly, we also observed IgV_H-CDR3 amino acid homology among different RS B-CLL. *RS8* expressed a V_H3-30/D3-22/J_H6 rearrangement and the IgV_H-CDR3 amino acid sequence showed ≥75% homology to the CDR3 sequence of RS *case 3* of which the V_HDJ_H rearrangement was unfortunately not described (29). In addition, the IgV_H-CDR3 amino acid sequence of *RS8* showed 68% homology to the IgV_H-CDR3 amino acid sequence of RS *3557* (30) although it must be noted that the latter expressed a V_H3-74/ D3-09/J_H6 rearrangement (Figure 1).
RS4 showed IgV_H-CDR3 amino acid homology (≥75%) with RS *B-CLL57* (31). *RS4* and RS *B-CLL57* not only expressed the same V_HDJ_H rearrangement (i.e. V_H4-39/D6-13/J_H5) but also the same Vκ012/02 -Jκ1 gene rearrangement (data not shown). Based on the IgV_H-

CDR3 homology that is observed between B-CLL, 8 homology groups have been defined (10). *B-CLL57* is an IgG$^+$ B-CLL that shows homology with 4 other unmutated V_H4-39 expressing IgG$^+$ B-CLL (11). The IgV_H-CDR3 region of the first time point of *RS4* (*RS4a*) was homologous to 2 B-CLL of this homology group (*B-CLL57* and *B-CLL202*).

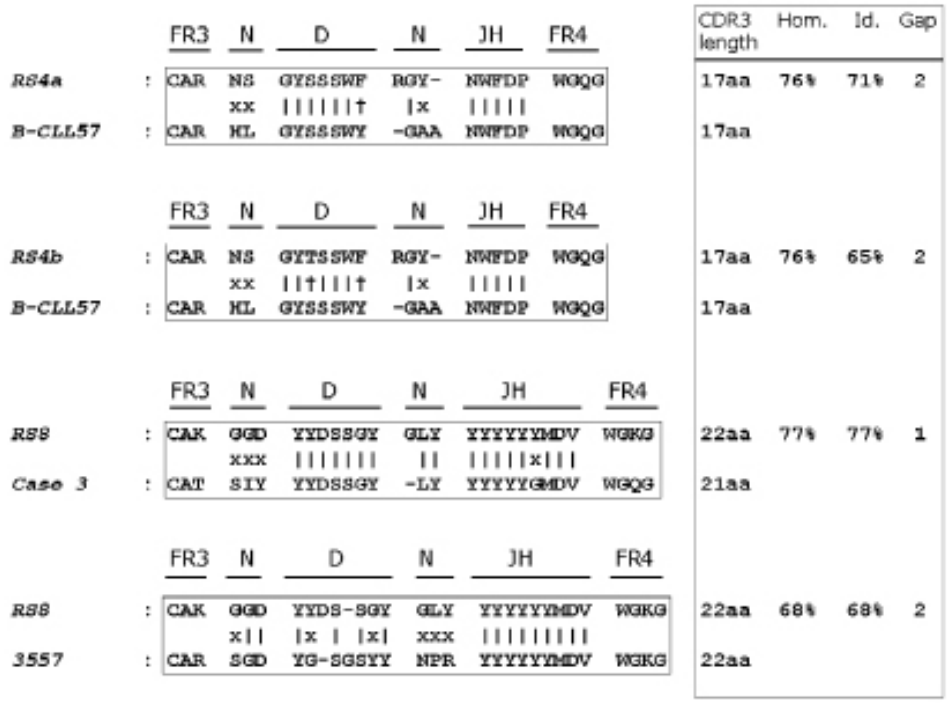

Figure 1. IgV_H-CDR3 amino acid sequence homology of *RS4* and *RS8* with IgV_H-CDR3 of 3 previously described RS B-CLL. The IgV_H-CDR3 amino acid sequence of *RS4* is homologous to the IgV_H-CDR3 amino acid sequence of *B-CLL57*. Both RS B-CLL expressed the same V_HDJ_H gene rearrangement. The IgV_H-CDR3 amino acid sequence of *RS8* is homologous to the IgV_H-CDR3 amino acid sequences of c*ase 3* and *3557*. Whereas *RS8* expressed the same V_HDJ_H gene rearrangement as *case 3*, it differed from the V_HDJ_H gene rearrangement of *3557*. Amino acids are depicted by the single letter code. FR3 and FR4 indicates framework region 3 and 4; N, amino acid encoded by the non-templated nucleotides; D, gene segment; JH, gene segment; | identical amino acid; +, similar amino acid; Hom, percentage of homologous amino acid; Id, percentage of identical amino acid; Gap, length difference in amino acid of the compared IgV_H-CDR3 sequences.

Remarkably, due to an extra mutation in the IgV_H-CDR3 region, the DLBCL of *RS4 (RS4b)* shared homology with a total of 4 B-CLL of this CDR3-homology group (*B-CLL57*, *CLL202* and additionally *CL114* and *CLL209*) (11) (Figure 1). This B-CLL subgroup, previously denoted by us as homology group 6, is thus extended by our IgG$^+$ *RS4* and now includes a total of 6 B-CLL, 2 of which underwent Richter's transformation over time. In conclusion, these data demonstrate that Richter's transformation occurs preferentially in unmutated

B-CLL. Furthermore, we found that the most hypervariable region of the Ig gene, the CDR3 region, was highly homologous among distinct RS B-CLL cases.

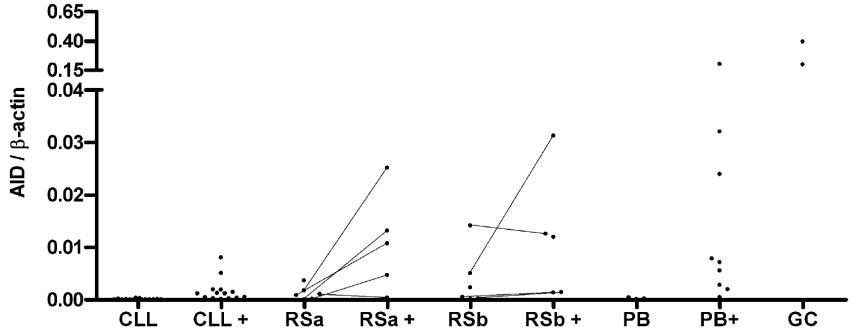

Figure 2. Relative AID expression levels of RS B-CLLand control B-CLL before and after stimulation. Quantitative RT-PCR analysis of AID and β-actin was performed on peripheral blood samples of a panel of 15 B-CLL without reported transformation and 5 B-CLL that transformed to a DLBCL. To induce AID expression, the B-CLL and RS samples were cultured for three days in the presence of IL4 and CD40L. RSa indicates the tumor sample before Richter's transformation and RSb indicates the tumor sample after transformation to a DLBCL. Each dot represents the average value of at least three AID / β-actin ratio measurements.

Endogenous and induced expression of Activation-induced cytidine deaminase.

Next, we analyzed the role of the somatic hypermutation machinery during Richter's transformation. To this end, we quantitatively measured the expression of AID in 5 RS B-CLL before and after transformation and compared these to the expression levels of peripheral blood samples of 15 control B-CLL and of sorted tonsillar germinal center B-cell fractions (Figure 2). AID expression was not quantifiable in any of the 9 (3 IgM^+, 6 IgG^+) mutated B-CLL, nor in 6 unmutated B-CLL (IgM^+). Interestingly, 4 of 5 RS B-CLL did express measurable levels of AID, although the AID/β-actin ratios were clearly below the ratios observed in germinal center B cells (Figure 2).

It has been described that CD40 engagement induces AID expression in B cells(25, 32). To investigate if the malignant cells were still responsive to environmental stimuli with respect to their AID expression, 3 RS B-CLL (*RS3*, *RS4*, *RS6*) and 14 control B-CLL were cultured for three days on either untransfected or CD40L-transfected L cells in the presence of IL4 and anti-IgM coupled sepharose beads. As positive controls, peripheral blood B cells and an EBV B cell line were used. Under these conditions, AID expression was increased in healthy donor peripheral blood B cells, the EBV B-cell line (data not shown) and in the mutated (3 IgM^+ and 6 IgG^+) and unmutated (6 IgM^+) B-CLL. However, the stimulated RS B-CLL expressed significantly higher levels of AID, both before and after transformation, as compared to both control B-CLL groups (Figure 2).

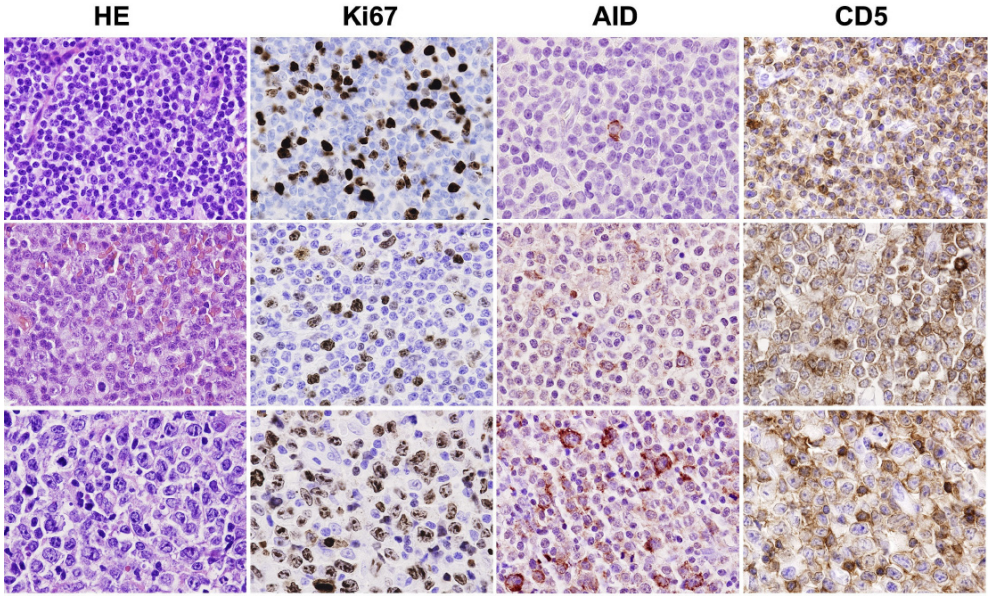

Figure 3. AID protein expression in *RS8* and control B-CLL. Haematoxylin&Eosin, Ki67, AID and CD5 stainings on lymph node material of an unmutated B-CLL (upper panel), *RS8a* (middle panel) and *RS8b* (lower panel). Small B-CLL cells are in all cases negative for AID, whereas proportions of blastoid cells of *RS8a* and *RS8b* show clear cytoplasmic AID staining. Magnification 25x.

To further investigate the role of AID in Richter's transformation, the expression of this protein was visualized immunohistochemically in paraffin-embedded tissue sections of two RS patients (*RS1* and *RS8*), of 11 lymph node samples of control B-CLL and of a tonsil (Figure 3). In the latter, AID was found within the germinal center blasts and in scattered extrafollicular centroblast-like cells (not shown), as was previously reported by Greiner *et al* (22). In accordance with the mRNA expression data of blood-derived B-CLL samples, in 9 out of 11 B-CLL lymph node specimens no AID-expressing cells were present. In one unmutated control B-CLL, sporadic AID-expressing paraimmunoblasts were found in some proliferation centers (Figure 3, upper panels). In another control B-CLL, more AID-expressing centroblast-like cells were observed. However, since in this biopsy scattered residual germinal centers were present we could not exclude that these centroblast-like cells were GC related (data not shown). No QRT-PCR data were available on these patients to confirm these findings. In contrast to the control B-CLL and in accordance with the QRT-PCR data, AID-expressing cells were present in both RS cases of which lymph node material was available, i.e. *RS1* and *RS8* (Figure 3, middle and lower panel). It is noted that in both tumors, cytoplasmic AID expression was never observed in the small B-CLL cells but was confined to the centroblast-like cells. Immunohistochemical staining for BCL6, CD21 and BCL2 excluded the presence of residual germinal centers in the tissues (data not shown).

Somatic hypermutation in IgV_H and non-Ig genes in B-CLL undergoing Richter's transformation.

Since both quantitative RT-PCR and immunohistochemistry demonstrated that AID is expressed in RS B-CLL, we searched for evidence that the somatic hypermutation machinery indeed has been active in the tumor cells. Individual molecular clones of the amplified IgV_H genes of *RS1, RS3, RS4, RS6,* and *RS8* were sequenced. The degree of intraclonal sequence variation in IgV_H was compared with that of 6 unmutated and 9 mutated B-CLL without reported transformation. We found significant intraclonal variation (i.e. a mutation frequency higher than the Taq error rate determined in our laboratory) in several IgV_H mutated- and unmutated B-CLL and in 2 of the 9 RS B-CLL (*RS4a* and *RS6b*). The degree of intraclonal variation was low with a mean of 0.3 nucleotide differences per clone (Table 4). The observed nucleotide differences were present in only a minority of the clones. Finally, the nucleotide differences that accounted for the ICV in the RS B-CLL were non-confirmed and present in single clones only. The IgV_H genes are not the only genes that can be targeted by the somatic hypermutation machinery. Since *BCL6* and *PIM1* are described to be mutated in DLBCL as well (20), we amplified, cloned and sequenced these genes in selected RS B-CLL (Table 4). Of *RS1*, *RS3, RS4* and *RS8*, 790 basepairs (bp) downstream of the transcription initiation site of *BCL6* were analyzed. This region includes part of the first intron (position 358 to 1148 according to GenBank AY189709). In *RS4* and *RS8* one polymorphism (G → C) at position 754 was found in all clones. The consensus sequence of *RS1* already harbored one mutation (C → T) at position 897 before Richter's transformation. Interestingly, after transformation an additional (T → C) mutation was found in the consensus sequence at position 1075. Although *RS1* and *RS3* showed a low degree of intraclonal variation in *BCL6* (0.4 and 0.5 per 300 bp per clone respectively, all nucleotide alterations were non-confirmed and found in single clones only), this was significant and higher than the intraclonal variation observed in their IgV_H genes (≤0.2 per IgV_H gene). No intraclonal variation was observed in *BCL6* of *RS4* and *RS8*. For *PIM1*, 600 bp downstream of the transcription initiation site was analyzed in *RS1* and *RS8* (position 859 to 1623 according to GenBank AF386792). In both RS B-CLL the consensus sequence harbored a polymorphism (C → G) at position 1039. Neither mutations nor intraclonal variation were found in this region in either of these lymphomas. Taken together, quantitative RT-PCR and immunohistochemistry both demonstrated that AID is expressed in RS B-CLL. Furthermore we observed a low but distinct degree of ongoing hypermutation in either the IgV_H genes or *BCL6*, indicating that the hypermutation machinery indeed has been active during Richter's transformation.

Discussion

To our knowledge, a total of 97 transformed B-CLL have so far been described (27, 29, 30, 33-52). In 74 B-CLL patients (76%), the secondary lymphoma was classified as a DLBCL whereas in 23 B-CLL patients (24%) a Hodgkin's lymphoma developed. Overall, in 67 of the 97 RS cases (69%), the high-grade lymphomas were of the same clonal origin as their low-grade precursors. Clonality was in most studies assessed by southern blot analyses. IgV_H-CDR3 sequences of only 9 of these 67 RS B-CLL were available. Here we present 9 additional RS B-CLL in which the high-grade lymphomas were all of the same clonal origin as the preexistent B-CLL. Interestingly, of these altogether 18 RS B-CLL, 16 belonged to the unmutated subgroup, indicating that Richter's transformation occurs almost exclusively in this subset of B-CLL. It is noted that this finding does not necessarily account for the well documented poor prognosis of the unmutated B-CLL subset, since Richter's transformation is a rare phenomenon (53, 54).

Previously, several groups including ours have reported that the IgV_H-CDR3 amino acid sequences of a significant fraction of B-CLL are highly homologous, particularly within the group of unmutated B-CLL (7-11). This type of homology is unique for the group of B-CLL, and was not found within extensive cohorts of follicular lymphomas, DLBCLs, Burkitt's lymphomas and multiple myelomas (10). This suggests that a proportion of B-CLL recognizes recurrent antigenic epitopes. Of the 18 RS B-CLL analyzed in this study, the CDR3 region of as many as 8 (44%) displayed homology to IgV_H-CDR3 amino acid sequences present on GenBank. This frequency is not higher than that observed within the group of unmutated B-CLL (44%) (10). More remarkable is the finding that the IgV_H-CDR3 amino acid sequence of 5 of these 8 RS B-CLL (i.e. *RS4*, *B-CLL57* (11) and *RS8*, *case3* (29), RS *3557* (30)) exhibited inter-RS group homology. Both *RS4* and *RS8* express an unmutated IgG. In fact, all RS B-CLL with CDR3 regions homologous to that of *RS4* expressed unmutated IgG and all have a reported aggressive clinical course. Furthermore, whereas *RS4* shared CDR3 homology with 2 of such IgG^+ B-CLL before transformation, an additional mutation in the CDR3 region of *RS4* after transformation resulted in CDR3 homology with a total of 4 of these IgG^+ B-CLL. Altogether, these findings point towards selective forces that favor outgrowth and possibly also progression of tumor (sub)clones with B-cell receptors of restricted specificities. Identification of the antigens involved may further clarify the biological mechanism underlying tumor progression and provide tools for therapeutic intervention. Alternatively, IgV_H gene analyses may thus be of value to identify B-CLL with a poor biological behavior.

Our quantitative RT-PCR demonstrated that in peripheral blood samples of the control B-CLL AID levels did not exceed background levels. Our findings seem to contradict several papers reporting on AID expression in B-CLL (25, 55, 56). In most of these studies however,

AID mRNA expression was measured by non-quantitative RT-PCRs and by consequence the actual expression levels are difficult to judge. Limiting dilution assays revealed that less than 1% of the B-CLL cells express AID and accordingly quantitative AID mRNA measurements demonstrated that the expression levels found in the B-CLL were in all cases less than 5% of those found in GC cells (17, 55). In addition, western blot analyses showed that AID protein could not be detected in B-CLL regardless of their IgV_H mutation status (16). In contrast to our findings in B-CLL, AID expression was quantifiable in most of the RS B-CLL at presentation. After transformation, the AID levels varied considerably among the different RS B-CLL. Moreover, in the transformed stage AID expression seemed less influenced by *in vitro* CD40 stimulation, suggesting that the tumor cells are more autonomous. In general, the AID mRNA expression data were highly compatible with the observed AID protein expression in tissues as assessed by immunohistochemistry. In lymph node material of 9 of the 11 control B-CLL (mutated and unmutated), no AID expressing cells were observed, not even in the proliferation centers. In both RS cases that were histologically analyzed, scattered AID-expressing tumor cells were present. These AID-positive cells all had a blastoid appearance, whereas the small tumor cells were devoid of AID expression. It is not clear whether the AID-expressing cells are B-CLL cells activated by CD40L and IL4 or represent already transformed cells. In conclusion, our analyses indicate that in B-CLL the presence of significant numbers of AID-expressing cells is exceptional, whereas in RS B-CLL AID is more abundantly expressed and in fact may predict an aggressive clinical course.

Sequence analyses of IgV_H and *BCL6* demonstrated low but significant degrees of intraclonal variation in the RS B-CLL. In both genes, the nucleotide alterations were not equally spread over the different molecular clones, but clustered in a fraction of the clones. This suggests that the somatic hypermutation machinery is active in a minority of the B-CLL cells only, which fits the AID staining results. It is noteworthy that in two of the four RS B-CLL, the degree of intraclonal variation in *BCL6* was twice as high as the intraclonal variation observed in the IgV_H genes. Moreover, in the IgV_H-unmutated *RS1* an additional consensus mutation was obtained in *BCL6* during transformation, whereas the IgV_H gene remained unaltered. This finding is remarkable since in normal GC B cells the *BCL6* mutation rate is 10-100 times lower than the mutation rate in IgV_H (57-59). Mutations in *BCL6* have been reported in IgV_H-mutated (58-62) and IgV_H-unmutated B-CLL (63). It thus seems that at least in these RS B-CLL, the somatic hypermutation machinery, and most likely AID, can target *BCL6* (and potentially other non-Ig genes) while leaving IgV_H unaffected. This, together with the observation that AID expression, either spontaneous or induced, is higher in RS B-CLL and increases during transformation suggests a role for this genetic diversification mechanism during the ongoing transformation of the RS B-CLL.

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Precursor B lymphoblastic lymphomas originating from follicular lymphomas express hypermutated immunoglobulin heavy chains and surrogate light chains

Manuscript in preparation

Precursor B lymphoblastic lymphomas originating from follicular lymphomas express hypermutated immunoglobulin heavy chains and surrogate light chains

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Abstract

Follicular lymphoma (FL) is the prototype among germinal center-derived lymphomas and generally has a indolent clinical course. However, 20-60% of FL transform into high-grade lymphomas imposing dismal prognosis. Most often, transformation into other germinal center-type lymphomas, like diffuse large B-cell lymphoma or Burkitt-like lymphoma, occurs. Here, we describe three extraordinary cases of FL which progressed to $TdT^{+}CD20^{-}$ precursor B-lymphoblastic lymphomas (B-LBL). All three B-LBL had acquired a MYC translocation which was not present in the preceding FL. In one B-LBL, also the BCL6 gene was translocated, demonstrating that constitutive BCL6 expression not necessarily interferes with an immature B-cell program. Immunoglobulin analysis demonstrated that two of the FL expressed hypermutated IgG. Interestingly, the B-LBL expressed the same immunoglobulin heavy chains as their corresponding FL, including the hypermutated variable regions. This implies that the L-LBL had not evolved in parallel out of a pre-GC precursor cell carrying the BCL2 rearrangement. In addition, we obtained evidence for ongoing rearrangement of conventional light chains and expression of surrogate light chain expression in the L-LBL.

Introduction

Follicular lymphomas (FL) can be considered as the most highly differentiated exponents of germinal center (GC)-like B-cell non-Hodgkin lymphomas (B-NHL) [1]. The tumor cells not only cytologically and architecturally resemble normal GC B cells but also share the expression profile with that of GC centroblasts and centrocytes [2]. In accordance, FL express B-cell antigen receptors (BCR), encoded by the immunoglobulin heavy (*IgH*) and light (*IgL*) chain genes, with somatic gene modifications typical for the GC environment, i.e. variable (IgV) region hypermutation and class switch region recombination [3].

FL have a indolent clinical behavior with a reported median survival of 8 to 10 years after diagnosis [1]. However, 20-60% of FL undergo histological transformation to higher grade malignancies, which is associated with an aggressive clinical course, poor therapy responses and short survival [4;5]. Usually, transformation to other GC-type lymphomas, like diffuse large B cell lymphoma (DLBCL) or, less commonly, Burkitt-like lymphomas occurs. More surprisingly, these prototypic GC-like malignancies can also transform into precursor B-lymphoblastic lymphomas (B-LBL) [6-14]. Studies on incidental cases described so far indicated that the ensuing B-LBL carried a *BCL2* locus translocation, confirming the clonal relationship with the preceding FL. Karyotypic analyses showed a variety of chromosomal alterations gained in the B-LBL, among which *C-MYC* locus translocations were most consistent [6-9;12-14].

We here describe molecular analyses of three FL that transformed into B-LBL. We demonstrate that the evolved B-LBL phenotypically and functionally display key features of early B cells and that in addition to *C-MYC* also *BCL6* gene translocation may accompany this infrequent variant of FL progression. In addition, we address the potential mechanisms of tumor clone evolution and the question whether PAX5 gene alterations may be responsible for the cell program resetting in these GC-cell derived B-LBL.

Materials and methods

Clinico-pathological history of the three patients

TF 1: A 51 year old woman was diagnosed in 2002 with a FL grade I with tumor localization in cervical, axillary, mediastinal, peri-portal, para-aortic, inguinal regions, spleen and bone marrow (Ann Arbor stage IV). Treatment with chlorambucil resulted in regression of the lymphadenopathy. In 2003, a rapidly growing mass was detected anterior of the bladder. Biopsy of this mass demonstrated diffuse large cell non Hodgkin lymphoma (DLBCL) and treatment with cyclophosphamide/doxorubicine/vincristine and prednisone (CHOP) was started. Five months after the last course of chemotherapy, the patient was

admitted with dyspnoe. Upon examination a pericardial friction rub was noticed. Cardiac ultrasonic examination showed a large mass of the right ventricular wall reaching from the apex till the right atrium. A moderate circumferential pericardial effusion was found. The patient was transferred to another hospital for thoracotomy. Pericardial fenestrations were made to prevent progressive tamponade and peroperative biopsies of the right pericardial wall were taken. Pathological examination demonstrated localization of DLBCL. The patient was referred to our hospital for further treatment. Revision of the biopsies taken from the pelvic mass in 2003 revealed a lymphoblastic lymphoma instead of the initially diagnosed transformation to a DLBCL. The patient was treated with high dose chemotherapy consisting of iphosphamide/etoposide/methotrexate and two consolidation cycles with cytarabine/ etoposide/methotrexate. A complete remission was reached and maintenance therapy with mercaptopurine and methotrexate was initiated. A search for a matched unrelated donor was started, however, a few weeks later progression of the disease occurred and no further treatment was undertaken.

TF 2: A 55 year old woman was diagnosed in 2000 with a FL grade 1 with tumor localization in cervical, retroperitoneal and para-aortic regions (Ann Arbor stage IIIA). No therapy was initiated until december 2002, when she had complaints of progressive back pain. Within a few weeks, the patient was unable to walk and became incontinent for urine. She was admitted to our hospital and a magnetic resonance imaging (MRI) scan of the spine demonstrated multiple lesions and a fracture of vertebral body L3 with spinal cord compression. Laboratory evaluation showed 9% blasts in the peripheral blood and the bone marrow aspiration revealed 90% lymphocytic blasts, indicative for the diagnosis acute lymphoblastic leukemia (pre-B cell ALL). Lumbar puncture demonstrated leptomeningeal infiltration A computed tomography (CT) of the chest and abdomen showed multiple enlarged para-aortic and para-cava lymph nodes. Because of the spinal cord compression and progressive paresis, a laminectomy of L3 and L4 was performed. A peroperative biopsy of L3 demonstrated infiltration with lymphocytic blasts. Treatment was started with daunorubicin, vincristine, prednisone, asparaginase and an Omaya reservoir was placed for repeated intrathecal methotrexate administration. After the first cycle complete remission was obtained. Consolidation therapy consisted of cytarabin/mitoxantrone, followed by high dose methotrexate/ asparaginase/ mercaptopurine. The therapy was complicated by pneumocystis carinii pneumonia, CMV reactivation and urinary tract infection with Klebsiella pneumoniae and enterococcus species. Three months after she left the hospital the back pain recurred. Bone marrow aspiration revealed more than 20% blastic cells. The patient was treated with dexamethasone but died a few weeks later.

TF 3: A 23 year old woman was diagnosed in 2003 with a FL grade I with localization in cervical and axillar regions. No further information about this period could be retrieved but

probably no treatment initiated. In September 2005, the patient was admitted to a hospital with enlarged and painful cervical lymph nodes. Further evaluation demonstrated lymphoma localization in cervical, axillar, mesenterial, retroperitoneal and inguinal regions. Laboratory evaluation showed a leukocyte count of 10.7 x 109/L with 38% atypical lymphocytes. Immunophenotyping demonstrated two lymphocytic populations i.e. one population of mature monoclonal B cells and a dinstinct population of lymphoid blasts. There was infiltration of the cervix uteri. The patient was 4 months pregnant at that time and had a history of 3 previous spontaneous abortions. The pregnancy was terminated and combined chemotherapy (cytarabin/etoposide/methotrexate) was started. Complete remission was established following two additional courses of vincristin/dexamethason/adriamycine. Consolidation therapy consisted of cytarabin/asparaginase. Shortly thereafter the disease relapsed with large abdominal lymph nodes and progressive bilateral hydronephrosis. Salvage therapy with cyclophosphamide, doxorubicin and prednisone followed by etoposide/mitoxantrone was initiated and was complicated by grade 4 mucositis. Despite an initial response the disease relapsed again with progressive cutaneous lesions. Local radiotherapy was given. The patient died a few weeks later.

Patient material.

All lymphomas were classified according to the WHO classification system [1]. Part of the tumor material of *TF1, TF2* and *TF3* was freshly frozen in liquid nitrogen directly after surgical removal, and in part fixed in formalin and embedded in paraffin. This study was conducted in accordance with the ethical standards in our institutional medical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, revised in 1983.

Immunohistochemistry.

Immunohistochemical stainings were performed on acetone-fixed cryostat sections and formalin-fixed paraffin-embedded tissue using the highly sensitive Powervision^{+} detection system (Immunovision technologies, Daly City, CA). Endogenous peroxidase activity of the cryostat sections was blocked with 0.1% NaN3 and 0.3% H_2O_2 in methanol. Visualisation of antibody binding was performed for the cryostat sections with 3-amino-9-ethylcarbazole (Sigma, St Louis, MO), 0.03% H_2O_2 in Tris-HCl, pH 7.6. The sections were counterstained with hematoxylin (Marck, Darmstadt, Germany). Monoclonal antibodies specific for CD20 (B-Ly1), CD21-L (DRC-1), BCL2 (124), BCL6 (PG-B6P), CD79a (HM57), CD19 (HD37), Igλ (A193) (all from Dako, Glostrup, Denmark), CD10 (CALLA) and PAX5 (clone 24) (both from Becton and Dickinson, Erembodegem-Aalst, Belgium), CD3 (SP7; Labvision, Neomarkers, Fremont, CA) were used. A polyclonal serum directed against TdT was used

(Klinipath, Duiven, The Netherlands). CD19 was stained on cryostat sections, all other stainings were performed on paraffin-embedded material.

DNA isolation, RNA isolation and cDNA synthesis. RNA was isolated using the Trizol reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's description. First-strand complementary DNA (cDNA) was synthesized as described previously albeit 5'-$(dT)_{14}$-d(A/G/C)-d(A/G/C/T)-3' primers were used [15].

Amplification, cloning and sequencing.

PAX5 was amplified using the following primers 5'AATTATCCGACTCCTCGGAC3' in combination with 5'CTCCTGAATACCTTCGTCTC3' and 5'GAGACGAAGGTATTCA GGAG3' in combination with GGCAGCGCTATAATAGTAGG3'.

VpreB was amplified using the primers 5'CTCCTGTCCTGCTCATGCAC3' and 5'CTCATGCACTTTGTCTACTG3', *Lambda 5* was amplified using the primers 5'CTCCTGTCCTGCTCATGCTG3' and 5'GTACACACCGATGTCATGGTCG3'. IgV_H transcripts were amplified using a mixture of forward primers located in the FR1 regions of the IgV_H gene families V_H1 to V_H6 or alternatively in the FR3 region of V_H1 to V_H6 [16] in combination with one of the FAM-labeled reverse primers located in Cμ, Cδ, Cα or Cγ regions [17]. All PCRs performed in this study followed the same protocol. PCR mixtures contained 1x *Taq* buffer (20mM of Tris-HCl, 50mMof KCl, pH8.4), 2U Taq polymerase (Invitrogen), 1.5mM $MgCl_2$, 0.2mM of each dNTP and 0.5mM of each primer. The PCR reactions were performed in the thermal cycler (PTC-100, MJ research Inc, Watertown, MA) and started with 6 minutes at 95°C, followed by 30 cycles of successively 30s at 95°C, 30s at 55°C and 30s at 72°C. The reaction was terminated for 6 minutes at 72°C. PCRs were analyzed on a 1% standard agarose gel (Sigma), with the exception of IgV_H amplicons that were run on an ABI PRISM 3100 automated sequencer in the presence of either 1pM ROX 500 or 1pM ROX1000 marker (Applied biosystems, Warrington, UK). IgV_H-PCRs were analyzed using the program Genescan analysis (Applied Biosystems). When a monoclonal tumor population was present IgV_H amplicons were cloned into the pTOPO-TA vectors and transformed into TOP10 bacteria according to the manufacturer's description (Invitrogen) and 8 to 16 clones were sequenced of each lymphoma. Sequencing on both strands was performed by an ABI PRISM 3100 automated sequencer (Applied Biosystems) using the big dye-terminator cycle-sequencng kit (Perkin Elmer Corporation). The consensus IgV_H sequence is divined as the nucleotide sequence that is shared by more than 50 % of the clones. The intraclonal variation (ICV) was calculated as the mean number of nucleotide differences per clone compared to this consensus IgV_H sequence. PAX5 was cloned and sequenced as described above. To determine the *Taq* error rate of our experimental design, 48 clones of HPRT were sequenced using the primers 5'TTCCTCCTCCTGAGCAGTCAGC3' and 5'GCGATGTCAATAGGACTCCAGATG3'.

These clones were generated according to the same PCR and cloning procedures as used for the IgV_H genes. The *Taq* error frequency thus established is 0.2 per 300 bp. BCL2 PCR was performed on DNA as described previously [18].

Fluorescence in situ hybridization.

The presence of chromosomal aberrations was investigated in 4 µm thick paraffin-embedded tissue sections by fluorescence in situ hybridization (FISH). A segregation-detection assay was used to detect breaks in *BCL2* (probe Y5408), *C-MYC* (probe Y5410) according to the manufacturer's description (Dako). Breaks at 3q27 were also detected by split FISH, as described previously (probes RP11-137K3/5234 and 165I21/5227) [19;20].

Cytogenetic alterations.

Cytogenetic analyses was performed on metaphase cells after short term culture using trypsin-Giemsa banding technique. Chromosomal abnormalities are described according to the International System for Human Cytogenetic Nomenclature (ISCN 1985).

Results

Histology and immunophenotype.

Three FL, *TF1, TF2 and TF3* that underwent clinical and histological progression to a B-LBL, were analyzed in the study (Tables 1and 2).

Histologically, all three lymphomas displayed a typical follicular growth pattern. CD21-L and CD23 stainings confirmed that the tumor cells expanded in networks of follicular dendritic cells. All three FL consisted of mature, $CD20^+$, $CD79a^+$, $BCL2^+$ cells co-expressing the GC markers like CD10 and BCL6. BCL2 staining excluded the presence of reactive follicles in all three lymphomas. After transformation, the follicular growth pattern of *TF1* and *TF2* was lost and the tumor cells expressed CD79a, CD10 and BCL2, but no longer CD20. In addition, *TF1* expressed TdT after transformation. Of *TF2b,* approximately 10% of the tumor cells expressed TdT (2002), but this was no longer observed in *TF2c* (2003). In *TF3b*, part of the tumor cells retained a follicular growth pattern (2005), alternating with sheets of lymphoblastic cells (Figure 1). Like in *TF1b* and *TF2c*, these lymphoblasts lacked the expression of BCL6, CD20 but did express TdT.

Table 1. Clinico-pathological information of three patients with FL transforming into B-LBL.

Patient	gender	age	diagnose	year	location
TF1a	F	57	FL	2002	LN
TF1b			B-LBL	2004	epicard
TF2a	F	51	FL	2001	LN
TF2b			B-LBL	2002	mouth
TF2c			B-LBL	2003	mouth
TF3a	F	24	FL	2003	LN
TF3b			FL/B-LBL	2005	LN

B-LBL, precursor B lymphoblastic lymphoma; F, female; FL, follicular lymphoma; LN, lymph node.
In *TF1b* and *TF2,b* the pre-existing FL was no longer present, whereas in *TF3b* both the FL and B-LBL were detected. The biopsies of *TF2b* and *TF2c* were both taken from the mucosa of the jaw at location 2.7

Table 2. Immunophenotype of lymphomas before and after progression.

		Immunohistochemistry										PCR	
Patient	Diagnose	CD20	FDC	CD10	BCL6	BCL2	TdT	Pax5	CD79a	CD19	Igλ	VpreB	λ5
TF1a	FL	+	+	+	+	+	-	+	+	+	+	-	-
TF1b	B-LBL	-	nd	+	-	+	+	+	+	+	+	+	+
TF2a	FL	+	+	+	+	+	-	+	+	nd	nd	-	-
TF2b	B-LBL	-	nd	+	10%	+	10%	+	+	+	5%	nd	nd
TF2c	B-LBL	-	nd	+	+	+	-	+	+	+	+	+	+
TF3a	FL	+	+	+	+	+	-	+	+	nd	nd	nd	nd
TF3b	FL	+	+	+	+	+	-	+	+	+	-	nd	nd
	B-LBL	-	-	+	-	+	+	+	+	+	-	nd	nd

B-LBL, precursor B-cel lymphoblastic lymphoma; FL, follicular lymphoma; LN, lymph node; nd not determined. Some stainings were not performed due to lack of appropriate material. In *TF2b,* approximately 10% of all cells co-expressed BCL6 and TdT and 5% expressed Igλ. In *TF2c* nearly all cells expressed BCL6 and Igλ whereas no TdT expression was detectable.

Cytogenetics.

Segregation fluorescence in situ hybridization (FISH) revealed that *TF1a* and *TF3a,* in accordance with the immunohistochemistry data, both harbored translocations of the *BCL2* gene, which were still detected after histological transformation (Table 3). Unexpectedly, we

were not able to demonstrate a *BCL2* gene translocation in *TF2a,* although the BCL2 protein was highly expressed. After histological progression a *BCL2* translocation was detected in *TF2c.* In all three patients, *C-MYC* gene translocations were detected after histological transformation. In addition, 3q27 breaks involving *BCL6* were detected and showed a more complex pattern. Using two different probe sets, a 3q27 break was detected in *TF1a,* whereas after progression this chromosomal breakage was no longer detected. This was in accordance with the immunohistochemistry, revealing BCL6 protein expression in the FL of *TF1a*, but not in the B-LBL of *TF1b*. In contrast, *TF2c* showed a 3q27 break only after transformation and BCL6 protein was expressed both before and after transformation. In *TF3,* 3q27 gene breakage was detected at neither of the timepoints. Immunohistochemistry showed loss of BCL6 protein expression in the B-LBL of *TF3b* (Figure 1).

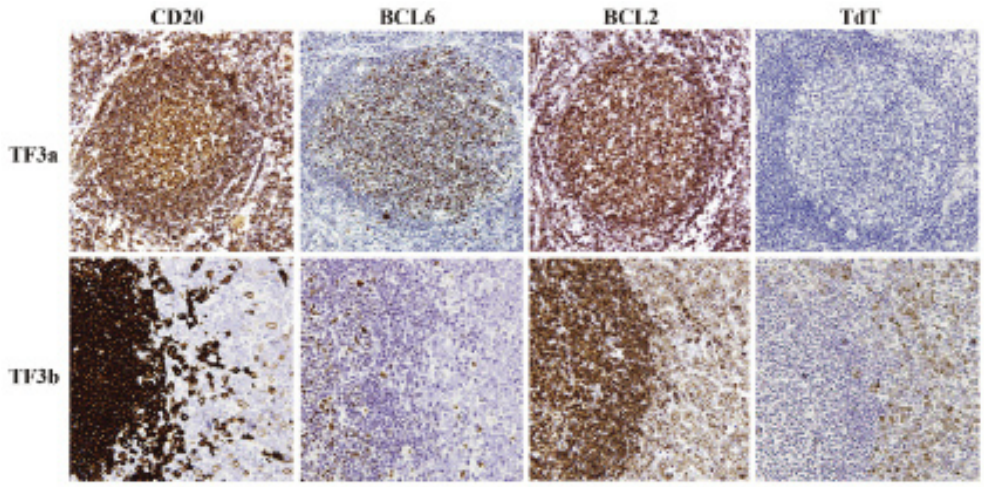

Figure 1: Immunophenotype and morphology of TF3 before and after transformation. Immunohistochemistry was performed as described in the materials and methods. TF3a showed a follicular growth pattern and expressed CD20, BCL6 and BCL2. After transformation part of the tumor cells of TF3b retained a follicular growth pattern, which was alternated with sheets of lymphoblastic cells. The follicular cells still expressed CD20, BCL6 and BCL2, whereas the lymphoblasts lost the expression of CD20 and BCL6 and expressed TdT instead.

Of *TF3b,* a karyogram was obtained from cell suspensions of the lymph node in 2005. Three tumor clones were analyzed of which one clone harbored the karyotype 46,XX,add(1)(p36),der(8)?t(8,18)(q24,q21)ins(8?)(q24;?) ,der(14)?t(8,14)(q22;q32), add(18)(q21). The second clone displayed the same karyogram with addition of a t(20,22)(q13;q1?1), whereas the third clone contained an additional copy of chromosome 7. All three clones harbored the t(8,14) (q22;q32), involving the *C-MYC* and *Ig* gene loci, indicating that they are subclones of the B-LBL.

Table 3. Chromosomal translocations detected by FISH analysis.

Patient	BCL2	C-MYC	BCL6
TF1a	+	-	+
TF1b	+	+	-
TF2a	-	-	-
TF2c	+	+	+
TF3a	+	-	-
TF3b	+	+	-

IgV_H gene analysis.

To establish the clonal relationship between the tumor populations at presentation and after histological transformation, the rearranged *Ig* V_HDJ_H genes were amplified by RT-PCR, cloned and sequenced (Table 4). In each of the three TF cases, the FL and B-LBL were monoclonal and clonally related. Sequence analysis revealed that, in accordance with their GC phenotype, the IgV_H genes of all three FL harbored somatic mutations. Remarkably, in *TF1 and TF2*, class switch recombination to IgG had taken place. *TF1a* presented with 25 nucleotide differences compared to the V_H gene of closest homology, i.e.VH4-61. Interestingly, the B-LBL of *TF1b* harbored 32 mutations of which 22 were shared with the dominant FL clone of *TF1a* (Figure 2). Comparison of the individual IgV_H clones showed a low but distinct intraclonal sequence variation (ICV) in the FL of *TF1a* but not in *TF1b*. However, at neither timepoints expression of activation induced cytidine deaminase (AID), the enzyme essential for SHM, was detectable by RT-PCR. The IgV_H genes of both *TF2a* and *TF2b*, harbored 24 identical mutations and, remarkably were also expressed as IgG. AID was not expressed and ICV was below taq error rate at both time points. Of *TF3a*, due to poor quality of the DNA, the IgV_H genes of the FL could only be investigated distal from the FR2. Still, 10 mutations were detected in the IgV_H and a low but distinct ICV was present. In the B-LBL of *TF3b*, 7 of these mutations were shared and 7 additional mutations were detected in the region proximal of CDR1. ICV was no longer present.

IgV_L gene analysis.

The FL of *TF1a* was found to express Vλ2-18/Jλ2(3a). Interestingly, after transformation a different monoclonal IgV_L rearrangement was amplified, i.e Vλ3-21/Jλ3b, which is located more upstream in the Ig lambda locus. Next, the lambda PCR was repeated on DNA followed by genescan analysis. In both *TF1a* and *TF1b* a biclonal IgV_L population was present, which corresponds to two rearranged Ig lambda loci. One of these clones was of the same length before and after transformation. However, in accordance with the IgV_L sequence analysis,

the second clone in *TF1b* was of a different length than the second clone of *TF1a*. It seems most likely that during transformation secondary rearrangement of the Ig light chain locus has taken place.

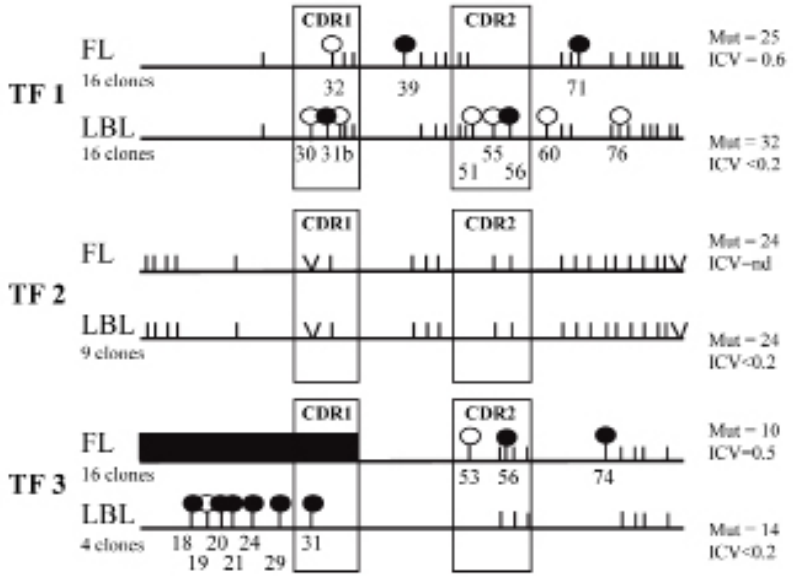

Figure 2: IgV_H configurations of FL and B-LBL. Schematic representation of IgV_H consensus sequences of *TF1, TF2* and *TF3* before and after progression. The vertical bars correspond to shared mutations between the FL and B-LBL. The lollipop-shaped symbols indicate nucleotide differences between the FL and B-LBL material. Replacement and silent mutations are indicated by closed and open circles respectively. Codon numbering is according to V-base. IgV_H gene mutation analysis of *TF3a* was performed using primers located in FR2. The black box thus denotes part of the IgV_H gene that is not sequenced. ICV denotes the degree of intraclonal variation.

RT-PCR, cloning and sequencing revealed that the FL of *TF2a* expressed Vλ1-36/Jλ3b. In *TF2b* however, this IgV_L rearrangement was not detected, and a non-clonal rearrangement pattern was seen. This was confirmed by DNA-PCR and genescan analysis. Cell suspensions of *TF3b* were sorted in $CD20^+$ $CD19^+$, $CD20^-CD19^+$, $CD10^+$ $CD19^+$ cell populations. Genescan analysis showed a monoclonal Igκ population of identical length in all three sorted cell populations.

The expression of the Ig light chain was also investigated by immunohistochemistry. Cryostat sections of the FL and precursor B-cell lymphoma of *TF1* and the precursor B-cell lymphoma of *TF2* were stained with antibodies directed against Igκ and Igλ. As was already shown by PCR, the tumor cells of both the FL and B-LBL of *TF1* clearly expressed Igλ and no Igκ. Unfortunately, due to a lack of cryostat sections of *TF2a*, IgL chain expression could not be investigated immunohistochemically. Nevertheless, in accordance with the IgV_L genescan and sequence analysis, in the B-LBL of *TF2b* no Igκ nor Igλ expression was detected.

Table 4. IgV_H gene and AID expression of three FL with progression to B-LBL.

Patient		IgV_H	mut #	ICV	IgL	Subclass	AID
TF1a	FL	V_H4-61/na/J_H5a	25	0.6	V_λ2-18/JL2/3a	IgG	-
TF1b	B-LBL	V_H4-61/na/J_H5a	32	< 0.2	V_λ3-21/JL3b	IgG	-
TF2a	FL	V_H3-20/na/J_H6b	24	nd	V_λ1-36/JL3b	IgG	-
TF2b	B-LBL	V_H3-20/na/J_H6b	24	< 0.2	polyclonal	IgG	-
TF3a	FL	V_H 1-69/D1-26//JH6c	10	0.5	nd	nd	-
TF3b*	FL	V_H 1-69/D1-26//JH6c	14	< 0.2	Igκ	nd	-
	B-LBL	V_H 1-69/D1-26//JH6c	14	< 0.2	Igκ	nd	-

nd, not determined # IgVH SHM was determined starting from FR1 in all instances, except for TF3a, in which it was determined starting from FR2. In TF3b, 14 mutations were found, of which 7 were located in FR1, and 7 distal from CDR1 which were also present in TF3a. In TF3a 3 mutations were present that could not be detected in TF3b.
* Peripheral blood of TF3b was sorted in 3 fractions, i.e. $CD20^+$ $CD19^+$, $CD20^-$ $CD19^+$, $CD10^+$ $CD19^+$. In all three fractions the same clonal rearrangement was detected.

Pre-B-cell receptor.

It has been described that precursor-B lymphoblastic lymphomas/leukemias in children and adults express the surrogate light chain (SLC) genes [21]. The SLC is composed of two proteins, VpreB and λ5. RT-PCR analyses revealed that both the B-LBL of *TF1b* and *TF2b* indeed expressed *VpreB* and *λ5,* whereas the preceding FL of *TF1a* and *TF2a* did not (Figure 3).

The role of PAX5 in the transformation of the FL into the B-LBL.

By immunohistochemistry it was observed that PAX5 was expressed in the FL and B-LBL of *TF1*, *TF2* and *TF3* (Table 2). Next, *PAX5* was amplified by RT-PCR, cloned and sequenced. No mutations were found in the *PAX5* gene (data not shown). Although the majority of the molecular clones contained full length *PAX5* including all exon sequences, a great variety of *PAX5* splice variants was found in *TF1* (Figure 4).

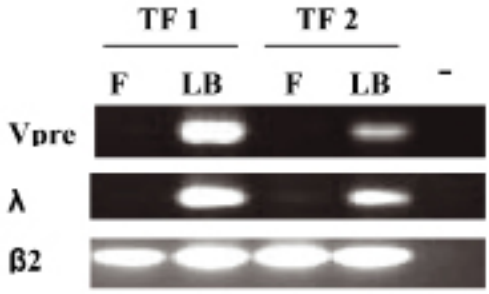

Figure 3: VpreB and lambda5 are expressed in the B-LBL but not in the FL. PCR analysis of *TF1* and *TF2* before and after progression.

In TF1a, splice variants lacking exon 2, exon 2 and 3, exon 4, exon 7, exon 7 and exon 8 and the 5' part of exon 9, exon 8 and the 5' part of exon 9, were detected. In *TF1b*, the majority of the clones also contained full length *PAX5* sequences, but again single molecular clones, lacking exon 2, exon 8 and the 5' part of exon 9 were detectable. In addition, two in frame splice variants, not described before, were expressed. One clone expressed 150 nucleotides of intron 4 between exon 3 and 4. One clone expressed 9 additional nucleotides between exon 7 and 8. Both new splice variants are submitted at GenBank. In *TF2*, lack of cDNA of the FL limited our sequence analysis to the B-LBL of TF2b. Here, next to the full length *PAX5* sequences, the splice variant lacking exon 8 and the 5' part of exon 9 was detected in single clones. To further asses the functionality of the PAX5 protein, the expression of two well known target genes of PAX5, CD19 and CD79a, was investigated immunohistochemically. Both lymphomas of all three patients indeed expressed these pan-B cell markers (Table 2).

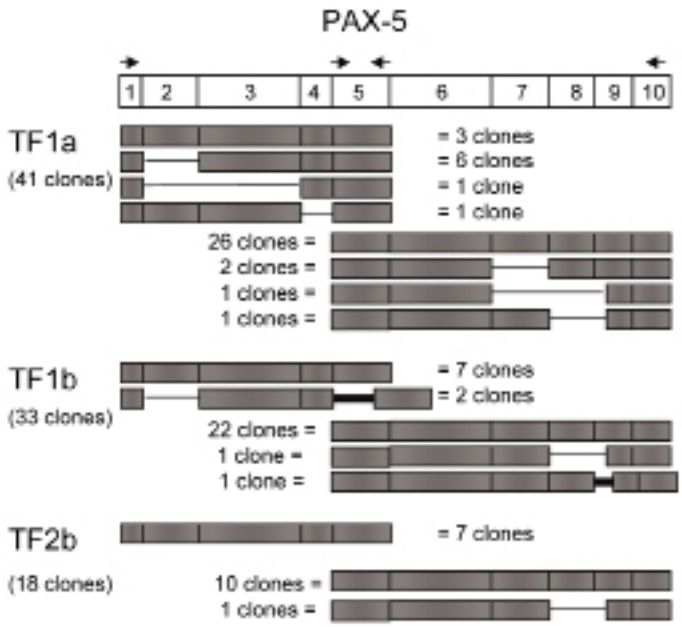

Figure 4: Expression of Pax-5 gene transcripts in TF1 and TF2. Schematic representation of the expression of different PAX5 mRNA splice forms in *TF1a*, *TF1b* and *TF2b*. Arrows denote primer localization. Grey boxes represent exons, thin lines represent deleted exons and thick lines insertions. The majority of the clones expressed full length PAX5. In *TF1a* splicevariants lacking exon 2, exon 2 and 3, exon 4, exon7, exon7 and 8, exon7 exon 8 and part of exon 9, and finally exon 8 and part of exon 9 were detected. In *TF1b* a splicevariant lacking exon 2, with an insertion of 150 bp between exon 4 and exon 5, was detected. as well as a splicevariant lacking exon 8 and part of exon 9, and finally a splicvariant with an insertion of 9 basepairs between exon 8 and exon 9. *TF2* could only be investigated after progression: In *TF2b* one splicevariant, lacking exon 8 and part of exon 9, was detected.

Discussion

Transformation of a FL to a B-LBL is a rare occurrence of which has been documented for sporadic cases before [6-14]. We here describe three B-LBL that evolved out of previously diagnosed FL and show that, in spite of retained expression of heavily mutated IgV_H genes, the B-LBL had acquired several key features of precursor B cells. The follicular growth pattern was lost, the pre-B cell specific genes TdT, VpreB and lambda 5 were switched on while the expression of CD20 was turned off. We also obtained evidence for pre-B-cell-specific Ig remodeling processes since the IgV_L genes in *TF1b* and *TF2b* were different from those in *TF1a* and *TF2a* respectively, while the IgV_H genes were unaltered.

The finding of C-MYC translocations in all three B-LBL, is in accordance with the literature. In 12 out of 14 (85%) reported cases of FL transforming into B-LBL, secondary *C-MYC* translocations were observed. For comparison, in FL that transform into a DLBCL not more than 10% were found to carry a secondary *C-MYC* rearrangement [22]. Rearrangement of the *C-MYC* gene thus seems more instrumental in the transformation of FL into B-LBL than into DLBCL. The t(14;18)(q32;q21) is typical for FL but rare in primary B-LBL/B-cell acute lymphoblastic leukemias (B-ALL). Stamatoullas *et al.* reported that of 142 adult B-ALL, only 5 had a translocation involving the *BCL2* gene [23;24]. Of note, the majority of t(14;18)(q32;q21)$^+$ B-ALL have complex chromosomal aberrations including translocations of both the *C-MYC* and *BCL6* loci [23;25]. To our knowledge a total of 19 t(14;18)(q32;q21)$^+$ B-ALL have been described thus far of which two had a preceding FL. Unfortunately, since IgV_H sequence analysis was not performed in the B-LBL it remains unclear of the other 17 B-LBL were transformed from a FL that was not detected due to rapid disease progression, or that they indeed arose as de novo precursor B-ALL. In *TF2,* progression to B-LBL was accompanied by a break at 3q27. *BCL6* region translocations have been described in 6-15% (grade 3) FL [26-28], ~ 30% of DLBCL and ~ 10% in FL transforming into DLBCL [4;29]. BCL6 is a transcriptional repressor important for GC formation [30]. Constitutive BCL6 expression results in a stop at this differentiation stage via the repression of *BLIMP1* [31]. The finding of continuous BCL6 expression in *TF2b* indicates that this blockage might be instrumental or at least does not prevent the switch to an early B cell program. *TF2b* harbors rearrangements of the *BCL2*, *BCL6* and *C-MYC* loci. This combination of genomic rearrangements has been reported before in sporadic cases of FL undergoing transformation to DLBCL as well as in de novo DLBCL [32;33]. Both the t(14;18)(q32;q21) and the t(8,14))(q22;q32) disrupt the Ig coding region [34;35]. The finding however that TF2b still expresses IgH mRNA is not necessarily contradictory since *BCL6* locus rearrangements may engage *IgL* chain or non-Ig loci, while *MYC* may be juxtaposed to downstream Ig switch regions thus leaving the Ig coding region intact [36]. Or alternatively, BCL2 and C-MYC may occur in the same IgH allele[37]. The observation in *TF1,* of a break at the 3q27 locus at presentation which was no longer detected in the B-LBL

of *TF1b* has been previously described also in conventional cases of FL transformation [4]. PAX5 is a pan-B cell transcription factor and a master regulator gene essential for B-cell commitment and differentiation [38;39]. PAX5 acts downstream of TCF3 and EBF and regulates the expression of e.g. CD19, CD79a, BLNK and CD72 [40]. Interstingly, Mullighan *et al*.[41] recently showed that approximately 30% of B-LBL have PAX5 mutations or copy number alterations i.e. mono-or bi-allelic losses, exon deletions or DNA amplifications of PAX5. Mono-allelic loss of PAX5 in B-progenitor ALL was accompanied by a two-fold reduction of PAX5 protein, indicative for haploinsufficiency [41]. *Pax5*-/- mice have a differentiation arrest at the pro-B-cell stage [42] whereas targeted deletion of *Pax5* in mature B cells results in loss of B-cell phenotype and lymphoblast formation [43]. These interesting observations prompted us to investigate if loss of PAX5 could have played a role in the cell-program resetting of the transformed B-LBL. However, we did not find mutations potentially affecting the coding region of PAX5. Our RT-PCR approach does not exclude mono-allelic losses of the PAX5 coding regions, but due to insufficient material we were unable to perform any quantitative mRNA or protein analyses. We did find clear expression of PAX5 and two of its direct target genes, CD19 and CD79, immunohistochemically, Moreover, all but two of the PAX5 mRNA splice variants that we detected have been described before in non-malignant B cells [44] and after transformation hardly any splice variants were detected. Taken together, since PAX5 was highly expressed both before and after transformation, as were at least two of its target genes, and no mutations were detected in PAX5, we believe it is unlikely that loss of PAX5 function was instrumental in the conversion of the FL *TF1, TF2* and *TF3* into B-LBL.

It has been hypothesized previously that clonally related FL and B-LBL do not develop sequentially but, alternatively evolve in parallel from a common pre-GC cell precursor harboring the t(14;18) [7;14]. According to this theory, the t(14;18)$^+$ precursor B cells may either, when engaged in a GC reaction in which they acquire additional hits, give rise to TdT$^-$ CD20$^+$ FL and/or by the GC-independent acquisition of genetic damage, including C-MYC translocation, become TdT$^+$CD20$^-$ B-LBL. The finding, in accordance with others [11], that the B-LBL of *TF1, TF2* and *TF3* harbored IgV_H mutations which were highly similar to those present in the preceding FL, demonstrate that the GC-independent route of B-LBL development is not occurring in these cases. However, in our opinion a third scenario cannot be excluded i.e. that the FL and the B-LBL do develop in parallel but out of t(14;18)$^+$ memory B cells. Roulland et al. [45] recently demonstrated that circulating t(14;18)$^+$ B cells in healthy individuals belong almost exclusively to the (either or not class switched) CD27$^+$ memory subset. In addition, we recently obtained evidence for repeated entry of memory B cells into the GC environment [46]. Future genetic profiling studies may reveal to what extent genetic aberrations between FL and the subsequent B-LBL are shared, thereby unveiling parallel or sequential pathogenetic pathways.

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Primary Follicular Lymphoma of the Small Intestine: $\alpha 4\beta 7$ expression and immunoglobulin configuration suggest an origin from local antigen-experienced B cells

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Primary Follicular Lymphoma of the Small Intestine: α4β7 expression and immunoglobulin configuration suggest an origin from local antigen-experienced B cells

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Abstract

Primary follicular lymphoma of the gastrointestinal tract (GI-FL) is a rare so far poorly studied entity. We analyzed four FL cases located in the small intestine and duodenum in order to gain insight in their pathogenesis and to find an explanation for their low tendency to disseminate outside the GI-tract. GI-FLs resemble nodal FLs with respect to morphology and expression of typical GC markers; CD10, CD38 and BCL-6. We established that the high levels of the anti-apoptosis protein BCL-2 in the tumor cells are in all cases due to a t(14;18) involving the immunoglobulin (Ig) heavy chain and *BCL-2* loci. Detailed immunoglobulin (Ig) gene analyses on microdissected tissue samples further supported the GC-cell derivation: GI-FLs carry extensively mutated variable heavy chain genes. The mutation patterns indicated that at some time point in development stringent antigen-receptor based selection processes must have occurred. Interestingly, three of four neoplasms expressed surface IgA, an immunoglobulin class typical of the mucosal immune system and seldomly found in nodal follicular lymphoma. Moreover, in contrast to nodal FLs, the GI-FLs expressed the α4β7 integrin, an established mucosa homing receptor also expressed by normal intestinal B and T lymphocytes and by low-grade mucosa-associated lymphoid tissue (MALT) lymphomas. The combined data suggests that primary FL of the small intestine is distinct entity that originates from local antigen-responsive B cells.

Introduction

In view of the size and activity of mucosa-associated lymphoid tissue (MALT), it is not surprising that 25 to 40 % of non-Hodgkin's lymphomas (NHL) arise at mucosal sites, most frequently in the gastrointestinal (GI) tract [1]. The most common GI-tract lymphomas are the classical low or high grade MALT B-NHLs [2,3]. Follicular lymphomas of the GI tract (GI-FL), by contrast, are rare with an estimated frequency of 1 to 3 % among the GI tract B-NHLs[4-6]. They occur most frequently in the small intestine, specifically in the duodenum[4-11]. In the lymph nodes, follicular lymphoma (FL) is one of the most common B-NHL and, by consequence, has been extensively studied. In its classical form, this neoplasm consists of follicular structures that harbor centrocytic and centroblastic tumor cells. These cells proliferate within networks of non-neoplastic follicular dendritic cells (FDC), similar to the GC B-cells of so-called secondary lymphoid follicles [12]. Like their normal counter parts, the tumor B-cells generally express CD10, CD38 and BCL-6 in addition to pan B-cell markers [12-14]. Nodal FLs most often express surface immunoglobulin M (sIgM) and sIgD, less frequently sIgG and rarely sIgA. At the molecular level, FLs are characterized by the t(14;18)(q32;q21) involving the Ig heavy chain (*IGH*) and *BCL-2* gene loci [15,16]. Due to this translocation, the oncogene *BCL-2* becomes constitutively expressed, preventing cells from apoptosis [17,18]. Molecular analyses of the variable (V) regions of *IGH*- and *IGL*- chain genes have further confirmed the germinal center (GC) origin of FLs: the IgV_H and IgV_L genes of FLs harbor significant numbers of nucleotide substitutions due to somatic hypermutation[19-21]. It is remarkable that in all the reported cases of primary duodenal FLs [5,6,8,9], including a large FL that invaded the pancreas[7], no evidence for distant or systemic disease was found. This low tendency to disseminate outside the GI-tract, which clearly contrasts the behavior of nodal FL [12], may be due to expression of specific adhesion molecules and/or dependence on local stimuli like, e.g., antigen or chemokines [22]. Mucosal lymphocytes strongly express the $\alpha4\beta7$ integrin while its ligand, MAdCAM-1, is selectively expressed on mucosal endothelium[23,24]. Accordingly, mucosa-associated B-NHLs, such as low-grade MALT lymphomas and mantle zone lymphomas presenting as malignant lymphomatous polyposis, have been shown to express the mucosal homing receptor $\alpha4\beta7$ [25,26]. In addition, it has been reported that intestinal epithelial cells produce a number of chemokines, *i.e.*, CLL25 (TECK) [27,28], CCL5 (RANTES)[29], CCL9 (MIG), CCL10 (IP10) and CCL11 (I-TAC) [30,31]. The respective receptors for these chemokines, CCR9, CCR5 and CXCR3 are expressed by $\alpha4\beta7^+$ T lymphocytes present in the lamina propria and in the epithelium [27,28,30-32]. Interestingly, it has recently been shown that CLL25 (TECK) also attracts IgA-secreting cells to the intestine [33]. Furthermore, CXCR3 is expressed by a small subset of peripheral B cells and by distinct types of B-cell malignancies such as low-grade MALT lymphoma, splenic marginal zone lymphoma and B-cell chronic lymphocytic leukemia (B-CLL)[34-36]. However, CXCR3 expression has not been

detected in nodal FLs [36]. Thus, expression of adhesion molecules and chemokine receptors determine homing and dissemination of normal and malignant B-cells. To explore to what extent FLs of the small intestine resemble their nodal counterparts and on the other hand to explain their localized nature, we performed a detailed analysis of the configuration of the expressed IgV_H chain genes and the expression of lymphocyte homing receptors in four cases of GI-FL. The results of these studies strongly suggest that these lymphomas are the offspring of local antigen-responsive B cells.

Table 1. Clinical findings and disease course of the GI-tract Follicular Lymphomas analyzed

Patient	Sex	Age	Localization	Stage*	Therapy	Time#	Outcome
1.	m	60	jejunum	I_E	resection	41	Alive, without disease
2.	f	68	duodenum	I_E	radiotherapy	60	Alive, without disease
3.	f	45	duodenum	I_E	radiotherapy	31	Alive, without disease
4.	f	35	duodenum	I_E&	chemotherapy	24	Alive, without disease

* Ann Arbor staging classification, # disease free survival time in months

& Positive t(14;18) on bone marrow samples but no histological evidence for bone marrow localization

Material and Methods

Patient Material

Fresh tissue material of the four gastrointestinal FLs, i.e. originating from a small bowel resection in one case and from endoscopically taken biopsies in three other cases, was in part snap-frozen in liquid nitrogen and in part fixed in formalin and paraffin-embedded.Patient no.1, a 60 year old male, was admitted because of an ileus with nausea and vomiting. Upon laparotomy, a stricturing tumor of 3.7 cm diameter was found which extended transmurally up to 1 mm from the serosa. Patients 2, 3 and 4 were females of 68, 45 and 35 years old which underwent endoscopic examinations for non-specific gastrointestinal complaints. In patient 2, a lesion was seen in the pars descendens of the duodenum covering an area of 3 cm diameter with a conspicuously nodular surface. A low-grade MALT B-cell lymphoma was suspected. In patient 3, a polypous tumor with a diameter of 1.5 cm was found in the area of the ampulla of Vater (Figure 2A). In patient 4 a lesion in the duodenum and focally in the ileum was found. In none of the four patients, histological evidence was obtained for systemic

disease. Patient 4 however, was treated chemotherapeutically based on demonstration of a t(14;18) by PCR on bone marrow. All achieved a disease-free status. The clinical data of the patients are summarized in Table 1. Tissue material of nodal FLs, MALT lymphomas, B-cell chronic lymphocytic leukemia and normal ileum, tonsil and lymph node were obtained from surgically removed specimens of our hospital.

Immunohistochemistry

The immunohistochemical stainings were performed on acetone-fixed cryostat sections and/or on formalin-fixed paraffin embedded sections using the highly sensitive Powervision $^{+}$ detection system (ImmunoVision Technologies, Daly City, CA). Endogeneous peroxidase activity was blocked of cryostat sections with 0.1% NaN_3, 0.3% H_2O_2 in PBS and of paraffin sections, after deparaffination and rehydration with 0.3% H_2O_2 in methanol. Visualization of antibody binding was performed for the cryostat sections with 3-amino-9-ethylcarbazole (AEC) (Sigma, St Louis, MO), 0.03% H_2O_2 in sodium acetate pH 4.9 and for the paraffin sections with 3,3'-diaminobenzidine (DAB) (Sigma), 0.03% H_2O_2 in Tris-HCl pH 7.6. The sections were counterstained with haematoxylin (Merck, Darmstadt, Germany). Monoclonal antibodies (MAbs) specific for CD10 (CALLA), IgM, κ- and λ- light chains (Becton and Dickinson, Erembodegem-Aalst, Belgium), MAbs specific for IgG, IgA, CD20 (B-Ly1), CD21-L (DRC-1, R4/23), BCL-2 (124) and BCL-6 (PG-B6P) (DAKO, Glostrup, Denmark), the MAb for CD38 (HIT2) (CLB, Amsterdam, the Netherlands), the MAb for CXCR3 (1C6) (Pharmingen, San Diego, CA) and the MAb for α4β7 (Act-1) [37] were used. MAbs for IgM, IgG, IgA, κ, λ, CD21-L, α4β7, CD10 and CD38 were only used on cryostat sections, MAbs for CD20, BCL-2, BCL-6 and CXCR3 were used on cryostat- and on paraffin- sections. For the CXCR3, BCL-2 and BCL-6 MAbs, the paraffin sections were pretreated with citrate buffer (10 mM pH 6.5) at 100 °C for 10 min.

Amplification & Analysis of t(14;18)

High molecular weight DNA was obtained from frozen tissue specimens by lysis in sodium dodecylsulphate (SDS) and 100 μg/ml proteinase K. The samples were digested at 56°C for 16 hours, followed by phenol-chloroform extraction and ethanol precipitation. After washing, the DNA samples were dissolved in distilled water. These genomic DNA samples were tested for the presence of the t(14;18)(q32,q21) using a PCR targeted at the *BCL-2*/*JH* breakpoint. The *BCL-2* mbr2 primer was used in combination with a reverse JH consensus primer JH18[38]. The PCR products were analyzed on a 1.5% agarose gel and subsequently purified. The purified PCR products were sequenced on both strands using the big dye terminator cycle-sequencing kit and an ABI sequencer (Perkin Elmer Corporation, Norwalk, CT).

Microdissection and cDNA Synthesis

Microdissection of groups of cells was performed with a PALM laser- microbeam system (Positioning and Ablation with Laser Microbeams; PALM GmbH, Bernried, Germany). Frozen tissue sections of 10 μm were mounted on plastic membranes and stained for 1 minute with haematoxylin. For RNA analyses, samples of approximately 50 cells were dissected out of tumor follicles and "catapulted" into 20 μl cDNA reaction mixture (see below) and kept on ice. Without prior RNA isolation, cDNA was synthesized employing 2 nmol of $Pd(N)_6$ primer (Pharmacia Biotech, Roosendaal, the Netherlands) and 160 units of M-MLV reverse transcriptase (Live Technologies, Breda, the Netherlands). The reaction mixture further contained 8 mM dithiothreitol (DTT), 1 mM of each dNTP, 1X First Strand Buffer (50 mM TrisHCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$) and 24 units of RNAse inhibitor (Boehringer Mannheim, Almere. the Netherlands). The reaction was performed for 15 min at 37°C after which the enzyme was inactivated during 10 min at 95 °C. After cDNA synthesis, 20 μl water was added.

Amplification of the V_H gene by PCR

The *IGV$_H$* locus and the applied primers in the PCR reactions are schematically depicted in figure 1. V_H family-specific PCRs were performed using different VH family-specific leader primers [39] in combination with reverse primers specific for either Cα (patient no.1 and 3) or Cμ (patient no. 2). (Cμ1: 5' CGTATCCGACGGGGAATTCTC 3'; Cα1: 5' TTCGCTCCAGGTCACACTG 3'). In the first round of amplification 1 μl of cDNA was used in a 25 μl PCR reaction volume. The PCR mixture contained 1X Taq buffer (20mM Tris HCL, 50 mM KCl, pH 8.4), 0.2 mM of each dNTP, 1.5mM $MgCl_2$, 1 unit of Taq polymerase (Life technologies) and 0.5 μM of each primer. First, 10 PCR cycles were performed in the thermal cycler (PTC-100, MJ research Inc., Watertown, MA) successively 30s at 95°C, 20s at 57°C and 20s at 72°C. The next 40 cycles of amplification consisted of 30s at 95°C, 20s at 55°C and 20s at 72°C. The reaction was completed for 6 min at 72°C. Under the same conditions, the complementary-determining region 3 (CDR3) was amplified in a nested PCR reaction using 2.5 μl of the first PCR product in a 25 μl reaction volume using a forward primer specific for framework region 3 (FR3) in combination with an appropriate nested reverse Cα or Cμ. primers [39]. The PCR products were analyzed on a 3% Methaphor agarose gel (FMC Bioproducts, Rockland, ME).

To obtain enough material for sequencing, the tumor-specific IgV_H products of first V_H family-specific PCR were amplified in a nested PCR using the appropriate VH-FR1-specific primer in combination with nested Cα or Cμ primers. Also here 2.5 μl PCR product of the first V_H family-specific PCR was used in a 25 μl reaction volume under the same PCR conditions (VH3-FR1: 5'-TCCCTGAGACTCTCCTGTG-3') PCR products were analyzed

on a 1 % standard agarose gel. The PCR products were sequenced on both strands. The IgV_H sequences found were compared with published germline IgV_H sequences using the Vbase database [40] and DNAplot [41] on the internet (http://www.mrc-cpe.cam.ac.uk) to identify somatic mutations. The amino acid sequences of the CDR3 regions were analyzed using the NCBI Protein-BLAST program, option "Search for short nearly exact matches" (http://www.ncbi.nlm.nih.gov/BLAST).

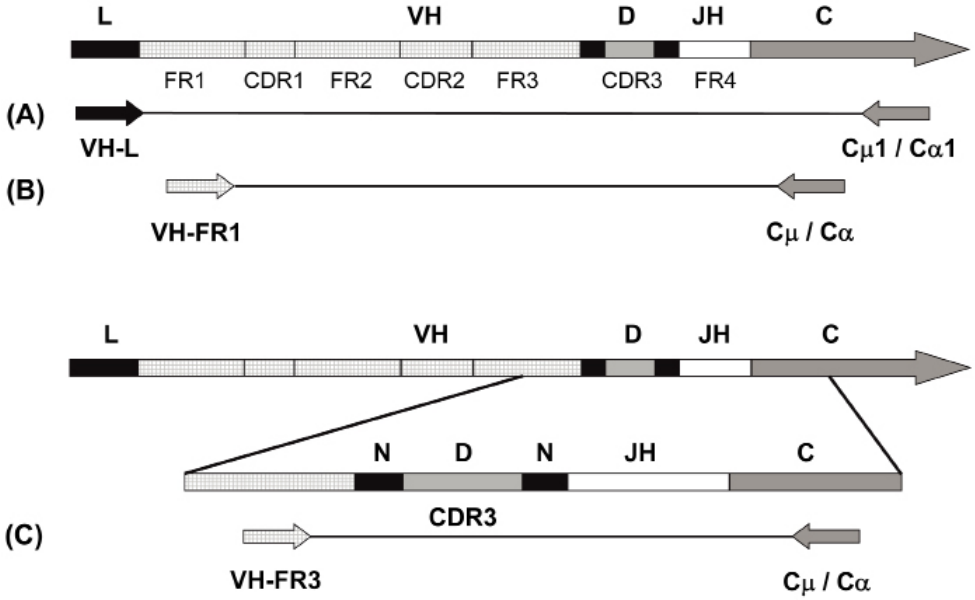

Figure 1. Schematic representation of the IgH locus and the primers used in the PCRs on the follicular lymphomas. L, leader sequence; V_H, variable gene segment; D, diversity gene segment; J_H, joining gene segment; C_H, constant gene segment; N, non-templated nucleotide additions; FR, framework region; CDR, complementarity determining region. (A) V_H-family-leader specific PCR; To amplify the V_H gene, V_H-family specific primers annealing in the leader regions were combined with downstream primers specific for the constant regions of the immunoglobulin gene, i.e. either Cμ1 for IgM-, and Cα1 for IgA-expressing lymphomas. (B) Nested V_H-family-FR1 PCR; The V_H region was amplified out of the products of PCR-A using nested V_H-family specific FR1 primers and Cμ and Cα downstream primers. (C) Nested CDR3-specific PCR; The CDR3 regions were amplified out of the products of PCR-A, using a consensus FR3 primer and nested Cμ or Cα downstream primers

Statistical Analysis

To calculate whether there is significant selection against replacement (R) mutations in the framework regions (FR), we used the binomial distribution model as proposed by Chang and Casali [42] and the multinomial distribution model as proposed by Lossos et al.[43]. Because the framework regions are essential for the overall structure of the IgV region, in normal Ag selected B cells counterselection for R mutations in these regions occurs. This results in lower Replacement/Silent ratios in the FR regions (R/S < 1.5) than would be expected if mutations would occur by chance only (R/S = 2.9).

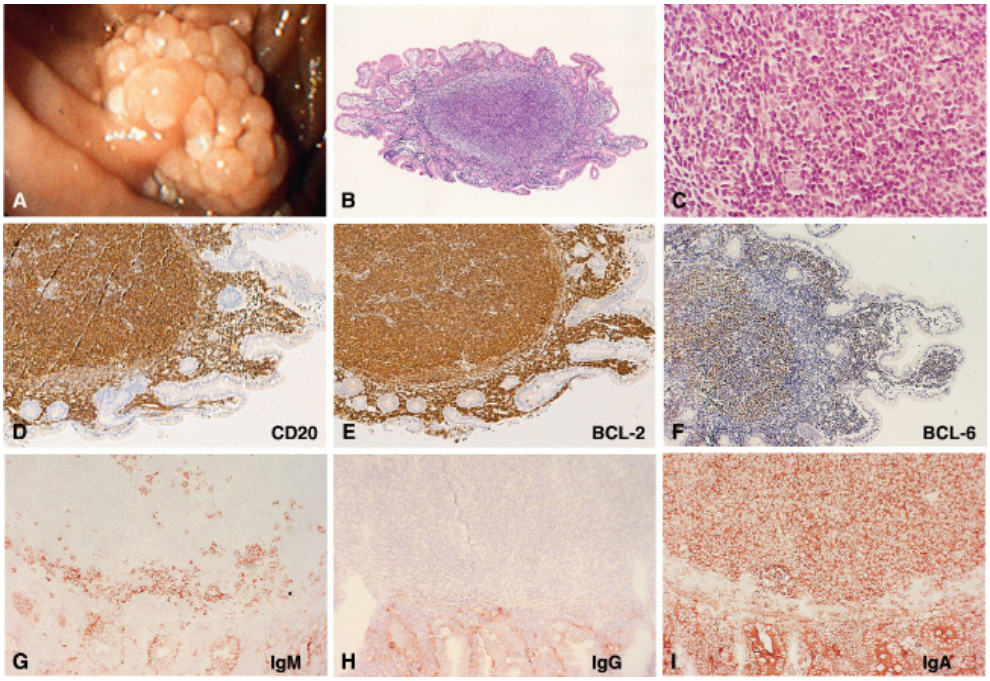

Figure 2. Endoscopy and histology of the duodenal polyp of patient 3. (A) Endoscopic picture of the adenoma-like structure found near the Ampulla of Vater. (B and C) Haematoxylin-eosin staining of a section through one of the tumor nodules showing a follicle-like lymphocytic infiltrate in the mucosa at 50X and 400X magnification, respectively. (D) CD20-staining, proving the B cell origin of the majority of the infiltrating lymphocytes. (E) BCL-2 staining showing strong overexpression of this oncogene by the B cells in the follicular infiltrates. (F) BCL-6 staining showing expression of this typical GC B-cell marker. (G-I) IgM, IgG and IgA stainings showing that the tumor cells express IgA exclusively

Results

Histopathological and Molecular Features of Primary Follicular Lymphomas of the Small Intestine

We studied four cases of primary FL of the small intestine (see Material and Methods). In patient 3, a typically polypous tumor with a diameter of 1.5 cm was found in the area of the ampulla of Vater (Figure 2A). Histologically, the tumors of all four patients consisted of dense infiltrates of predominantly small cleaved lymphocytes admixed with variable numbers of centroblasts and a few immunoblasts. The infiltrates displayed a clear nodular growth pattern reminiscent of normal lymph follicles (Figure 2B and 2C). Starry sky macrophages, which are prominent in reactive germinal centers, were absent. Unlike in classical MALT-type lymphomas, the lymphoid cells did not infiltrate and destruct the gland epithelium, *i.e.*, no lympho-epithelial lesions were present. Immunohistochemistry demonstrated that the tumor

cells consisted of mature CD20$^+$ B cells, expressing the typical GC B-cell markers CD38, CD10 and BCL-6, which are also expressed by the vast majority of nodal FLs [12-14] (Figure 2D and 2F, Table 2). Interestingly, the tumor cells of patients 1, 3 and 4 were IgM$^-$ IgG$^-$ IgA$^+$ (Figure 2 G-I, Table 2). CD21-L (DRC-1) stainings demonstrated that the tumor cells expand mainly in networks of follicular dendritic cells (FDCs) (Figure 5, Table 2). However, BCL-2$^+$ BCL-6$^+$ IgA$^+$ tumor cells were also found scattered in the lamina propria (Figure 2E, 2F and 2I).), Like nodal FLs, all four GI-FLs were found to carry a t(14;18). The translocations in all cases involved the major breakpoint region (mbr), located in the 3' untranslated region of the *BCL-2* gene, adjacent to one of the JH gene segments of the *IGH* locus (Table 2 and data not shown) [15,16].

Table 2. Immunohistochemistry and t(14;18) PCR

	Immunohistochemistry								PCR
Patient No.	CD20	CD21-L	Ig isotype	light chain	BCL-6	CD10	CD38	BCL-2	t(14;18)
1	+	+	IgA	nc	+	+	+	+	+
2	+	+	IgM	κ	+	nd	+	+	+
3	+	+	IgA	κ	+	+	+	+	+
4	+	+	IgA	κ	+	nd	+	+	+

"+" indicates the reactivity of the monoclonal antibody with the tumor cells, except for the CD21-L staining in which it indicates the reactivity with the nonmalignant FDCs. nd, not done; nc, not clear

Ig V_H Gene Analysis on Microdissected Tumor Samples

We amplified the IgV_H regions out of almost pure samples of tumor cells microdissected from frozen tissue sections of the GI-FL of patients 1, 2 and 3. In these experiments, separate PCRs were performed applying six V_H family leader region specific primers in combination with appropriate reverse constant heavy chain region specific primers, being either Cα1, in lymphomas nos. 1 and 3 or Cμ1 in lymphoma no. 2 (Figure 1). On the PCR products thus obtained, not visible on agarose gel, a nested CDR3-specific PCR was performed using an FR3 region specific primer, which anneals just 5' to the D gene, combined with a nested Cα- or Cμ- region specific reverse primer (Figure 1). Of each GI-FL, these nested CDR3 PCRs yielded sharp bands on agarose gel only in the condition that a VH3 specific leader primer had been used in the first PCR (Figure 3A). As this finding was reproducible in multiple

tissue samples of each lymphoma we were confident that we had confirmed clonality and identified the IgV_H genes, with their respective CDR3 regions, that were expressed by the lymphomas. To obtain sufficient amounts of PCR products for sequencing, nested PCRs on the primary VH3 PCR products were performed using a 5'primer annealing in the FR1 region in combination with the nested Cα or Cμ reverse primers (Figures 1 and 3B). To ascertain that these nested IgV_H products indeed originated from the tumor clones, we also performed a semi-nested CDR3 PCR on them. In all three cases, the obtained CDR3 products had sizes identical to the original CDR3 amplimers (not shown). The amplified IgV_H genes were sequenced and analyzed. Of lymphoma 1, 2 and 3 we obtained V_H sequences out of 6, 2 and 6 malignant follicles respectively. GI-FL no.1 used the V3-11 gene in combination with the JH4b gene, GI-FL no. 2 used the V3-48 and JH4b genes and GI-FL no. 3 used the V3-7 and JH6 genes (Table 3). Corbett et al.[44] proposed stringent criteria for the assignment of D genes: at least 10 constitutive nucleotides of identity are required to confidently assign a D gene segment. According to these criteria, we could not determine a D gene used in any of the three cases.

Table 3. Ig Heavy chain gene sequence analyses of the Small Intestinal Follicular Lymphomas

Patient No.	Ig Isotype	VH family	closest V_H Germline gene	No. of mutations* (%)	JH gene
1	IgA	3	V3-11	40(17.7%)	4b
2	IgM	3	V3-48	32 (14.2%)	5b
3	IgA	3	V3-7	21(9.3%)	6b

*counted starting from codon 23 where the FR1 primer ends

Number of Mutations, Mutation patterns and CDR3 Amino acid Sequence Analysis

The GI-FLs of patients no.1, 2 and 3 all expressed extensively mutated IgV_H genes with respectively 40, 32 and 21 nucleotide differences in their V_H genes compared to germline VH3 genes of closest homology (Figure 4, Table 3). We assessed the distribution of replacement (R) mutations versus silent (S) mutations over the CDR and FR regions. In all three cases the R/S ratios found in the FR regions were lower than those of the CDR regions (Table 4). According to the statistical analysis of Lossos et al.[43], in all cases the number of R mutations in the FR regions were significantly lower ($p < 0.05$) than would be expected if the mutations had occurred at random and in the absence of selective forces (Table 4). These data indicate that in spite of the high number of somatic mutations the overall structure of the IgV_H and thus of the B cell receptor (BCR) was preserved in these lymphomas.

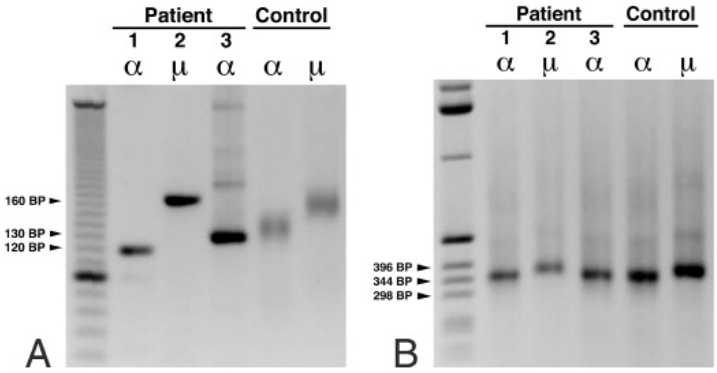

Figure 3. Nested CDR3- and V_H FR1-specific PCRs. (A) The products obtained by the V_H Leader-specific PCR, were subjected to CDR3 PCRs using the FR3 upstream primer and Cα (patient nos 1 and 3) or Cμ (patient no.2) downstream primers. These nested CDR3 PCRs on the VH3 PCR products yielded sharp bands of 120, 160 and 130 bp lengths on gel, proving the clonal nature of the proliferating cells of patients 1, 2 and 3, respectively. Control CDR3 PCRs on polyclonal B cells, shown in the last two lanes, yielded smear patterns on gel. (B) To be able to sequence the V_H products, the VH3-leader PCR products were subjected to nested PCRs using VH3-FR1 upstream primers in combination with Cα (patient nos 1 and 3) or Cμ (patient no.2) downstream primers. The VH3-FR1 PCR products obtained, were purified and sequenced. To give an impression of the lengths of the amplimers, the size of some marker-DNA bands are indicated.

It has been reported that the amino acid sequence of the CDR3 regions of 50 % of a panel of 20 gastric MALT-lymphomas showed significant homology to previously reported CDR3 sequences [45]. In two of these gastric MALT-lymphomas, as well as in the majority of salivary gland MALT-lymphomas [46], the CDR3 regions displayed at least 75% sequence homology with rheumatoid factors. Amino acid sequence analysis of the CDR3 regions of the GI-FLs presented here however did not reveal any resemblance to reported CDR3 regions, suggesting that the GI-FLs express unique CDR3 regions.

GI-FLs but not Nodal FLs Express the Mucosa Homing Integrin α4β7

Tissue sections of the intestinal and nodal FLs, MALT lymphomas, B-CLL as well as normal tonsil, lymph node and distal ileum (peyers patches) were stained with monoclonal antibodies specific for α4β7 and CXCR3, respectively (Figure 5, Table 5). Among the lymphomas, exclusively the GI-FLs and the low-grade MALT lymphomas expressed the mucosa-specific homing integrin α4β7. By contrast, and in agreement with our previous results [26], the vast majority of nodal FLs and the GCs of lymph nodes were α4β7 negative. In the normal ileum, the GCs displayed low expression of α4β7, whereas the mantle zone B lymphocytes and lamina propria T lymphocytes were strongly positive (Figure 5, Table 5). However, unlike MALT lymphomas, both the intestinal and nodal FLs expressed CD38 but lacked CXCR3 expression. Thus, the GI-FLs resemble normal GC B cells of tonsil and Peyers patches (Figure 5, Table 5). In agreement with Jones et al.[36], we found that 2 of the 3 tested cases of B-CLL were strongly CXCR3 positive (Table 5).

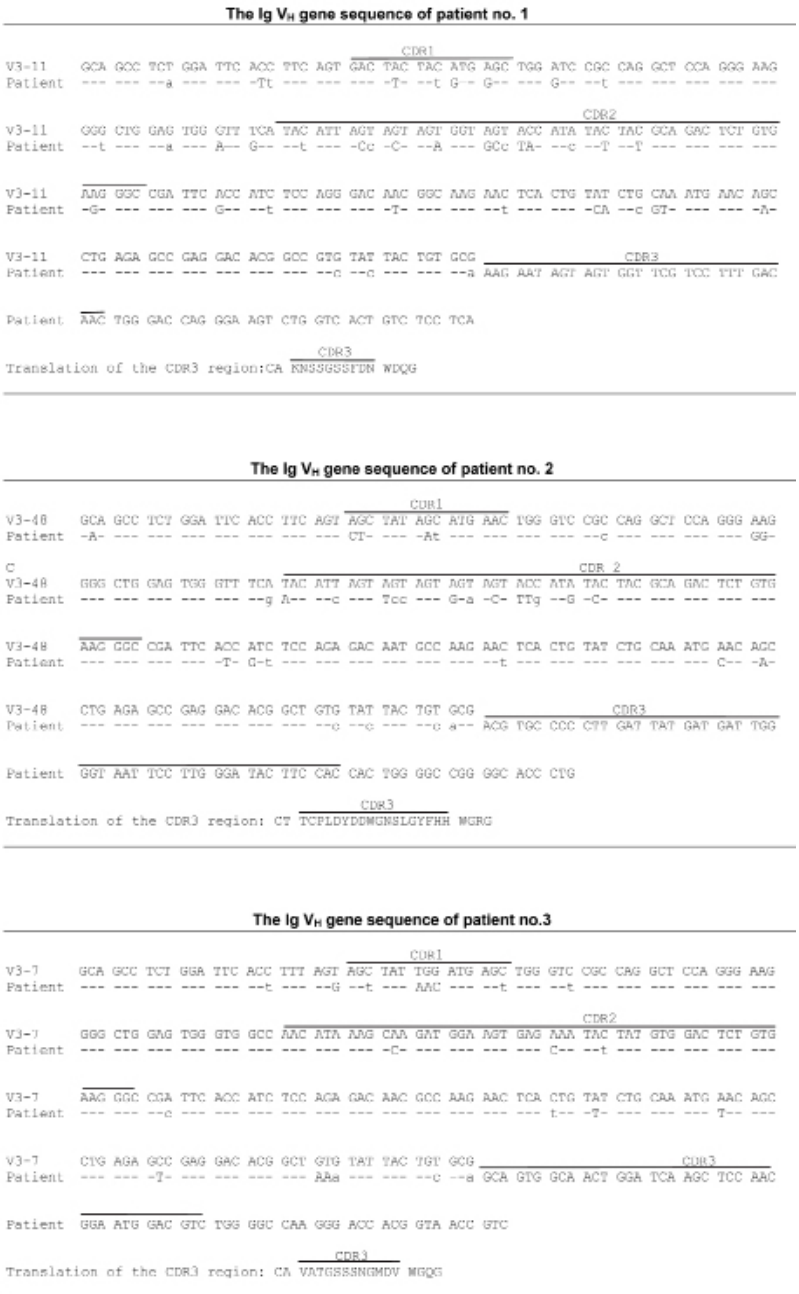

Figure 4. The IgVH sequences of the patients compared to the most homologous germline IgVH sequences. The individual complementarity regions (CDRs) are indicated with lines. Identity with the germline sequence is shown by dashes. Replacement mutations are indicated with uppercase letters and silent mutations are indicated with lowercase letters.

Table 4. Distribution of mutations in the IgV_H Genes of three GI-Follicular Lymphomas
**counted starting from codon 23 where the FR1 primer ends*

Patient No	Total No of Mutations*	Observed mutations in the CDR Regions			Observed mutations in the Framework Regions*				
		R	S	R/S	R	S	R/S	p Value[+]	p Value[#]
1.	40	13	5	2.6	11	11	1.0	**0.05**	**<0.05**
2.	32	12	5	2.4	8	7	1.2	0.14	**<0.05**

p Value; chance that the observed R/S ratio in the Framework Regions (<1.5) had occurred by chance only, i.e., in the absence of selective forces, [+]See Chang and Casali [42], [#]See Lossos et al. [43]

Discussion

In this paper we present four cases of GI-FL localized in the jejunum and the duodenum, the latter, according to previous reports [4-6], is the most frequent localization of this entity. In patient 1, the neoplasm had caused obstruction of the small intestine resulting in an ileus. In patients 2, 3 and 4, the lesions were relatively small, located in the duodenum and not evidently responsible for the patients' complaints. In accordance with other reports [5-9], the lesions had a conspicuously nodular surface. In patient 3, the appearance was even truly polypous and located near the ampulla of Vater. This localization has also been reported in five of eight GI tract FLs of a previous study and in four other cases [5,7-9]. It is also remarkable that 15 of the 22 duodenal FL patients reported so far [5-9] including our three duodenal FLs (2, 3 and 4), are female. Yoshino at al.[5] speculated that this might somehow be related to female predominance of bile duct diseases.

Cytologically and histologically the neoplasms were indistinguishable from their nodal counterparts. This morphological resemblance was supported by the immunohistochemical and molecular analyses. In addition to the pan B-cell surface protein CD20, the tumor cells expressed CD38 and BCL-6, markers typical of GC stage-derived B-cell malignancies and are generally not expressed by low-grade MALT or mantle cell lymphoma (Table 2)[12-14]. We established that the constitutive overexpression of BCL-2 is due to a t(14;18) also typical of nodal FLs [15,16,47]. This indicates that with respect to the earliest genetic alterations, the pathogenesis of nodal and GI-FL is similar.

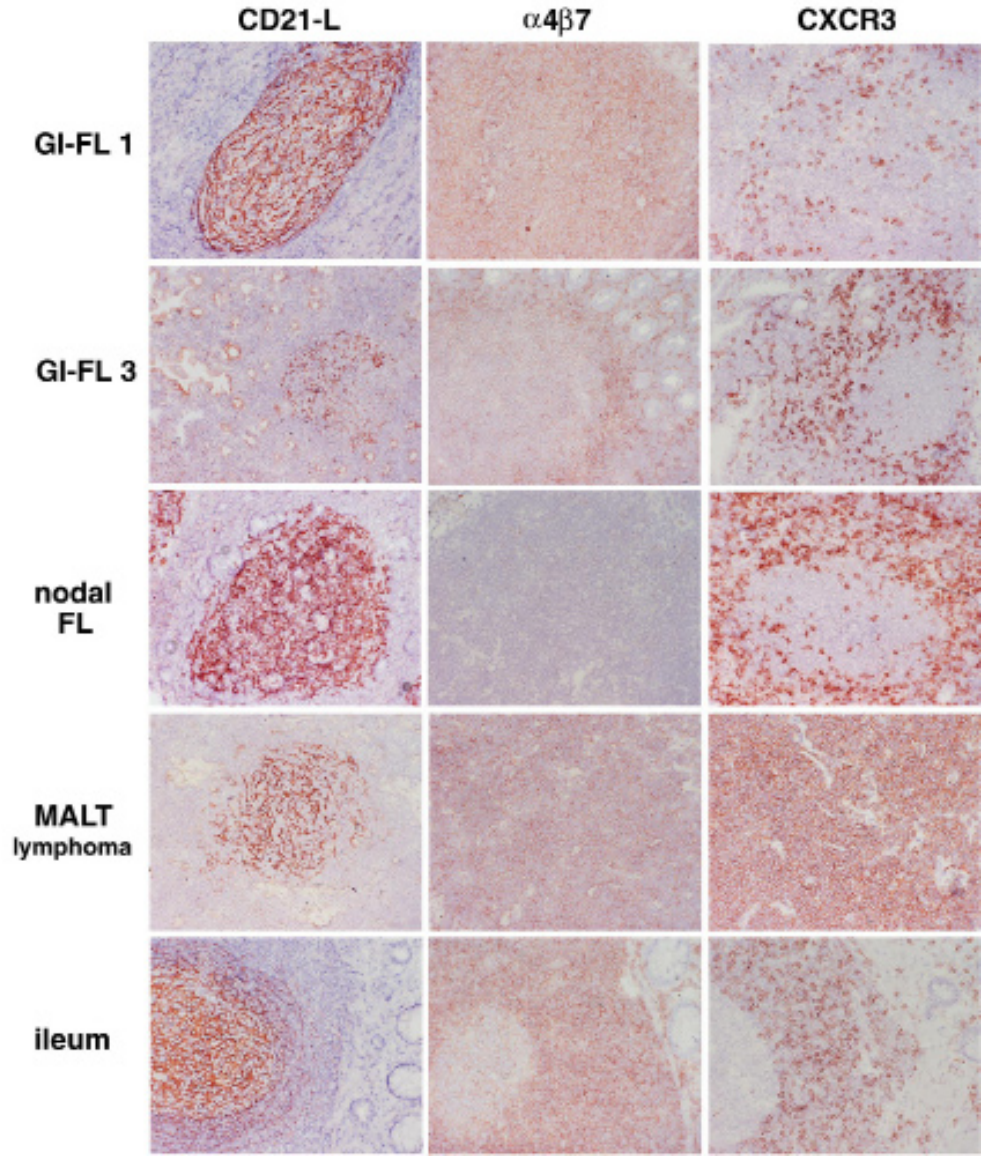

Figure 5. Immunohistochemical detection of CD21-L, α4β7 and CXCR3 of GI-FL, nodal FL, MALT lymphoma and normal ileum. In contrast to nodal FLs the GI-FLs express α4β7. Neither nodal FLs nor GI-FLs do express CXCR3. The T cells surrounding the malignant follicles are CXCR3 positive. Low-grade MALT lymphoma expresses both α4β7 and CXCR3. In normal ileum, all mantle-zone lymphocytes strongly express α4β7 and a significant fraction expresses CXCR3. The GC B-cells display low, but detectable α4β7 expression but no CXCR3 expression.

The Ig gene analyses demonstrated that all three analyzed GI-FLs carried heavily mutated IgV_H regions proving that the tumor cells indeed had undergone GC stage-specific alterations

(Figure 4, Tables 3 and 4). The mutation frequencies were significantly higher than those found in normal (post) GC B cells [21], compatible with a prolonged stay of the tumor cells in the GC environment. Moreover, we observed discrete nucleotide differences between molecular clones derived of each lymphoma (data not shown). Although this so-called intraclonal V gene diversity must be a reflection of the somatic hypermutation process. It is not certain to what extent this process continues during the tumor stage [20,48]. Detailed analysis of the observed mutation patterns indicated that, at least at some time of development, counterselection for potentially harmful replacement mutations must have occurred in the FRs. These patterns, physiologically found in normal antigen-selected B cells, suggest that expression of an intact B cell receptor (BCR) is also essential for the tumor cells to survive (Table 4).

It is intriguing that in three of our four GI-FLs analyzed, no evidence was obtained for distant or widespread disease and that these patients became disease free after local therapy only. In patient 4 chemotherapeutical treatment was given, based on the demonstration of a t(14;18) by PCR on bone marrow. The localized nature these neoplasms was most clearly illustrated in a patient described by Misdraji et al.[7] in whom, in spite of the fact that the duodenal FL had a significant volume and had invaded the pancreas, there were no signs of metastasis. In our study, the localized nature may be due to the fact that in at least 2 of the 4 cases (i.e. patients 2 and 3) the lesions happened to be diagnosed at a very early stage of disease. Still, this is in clear contrast to nodal FLs that are in majority systemic at the time of diagnosis, i.e. Ann Arbor stage III or IV [12]. Conversely, in spite of the systemic nature of the latter entity, the GI tract is not a frequent localization of primary nodal FLs. Thus supposedly, expansion of these neoplasms at mucosal sites depends on highly specific phenotypic qualities. In this respect, our observation that the GI-FLs differ from their nodal counterparts in that they express $\alpha 4\beta 7$, a well defined mucosal homing receptor which is specifically expressed by normal mucosal lymphocytes and by low-grade MALT lymphomas [26], is highly significant (Figure 5, Table 5). Also the lack of CXCR3 expression is compatible with their origin, since this chemokine receptor was not found to be expressed by normal GC B cells of the GI-tract either. MALT lymphoma, supposed to be derived of post GC B-cells, by contrast do express CXCR3 (Figure 5, Table 5).

Another explanation for their low metastasizing potential is invoked by the Ig analyses. The fact that three out of the four analyzed GI-FLs express IgA is of note, as this isotype is seldomly expressed by nodal FLs. In fact, this finding again indicates that these lymphomas may originate from local, antigen-responsive precursor cells, as IgA is the principal Ig class of the mucosal immune system (Table 2). This contention, obviously supported by the presence and distribution of somatic mutations in IgV_H, may imply that the BCRs expressed by these

FLs still have binding-capacity for antigens originating from the gut lumen. The localized nature of these GI-FLs may thus also be due to dependence on growth-supporting signals elicited by these BCR ligands potentially presented by FDCs in the tumor follicles. In this respect, nodal FLs seem different as their systemic nature suggests that they are independent of the presence of the original antigen. The group of primary GI- FLs may therefore be an attractive entity to study the concept of antigen-driven lymphomagenesis in humans.

Table 5. Expression of α4β7, CXCR3 and CD38

	Immunohistochemistry		
Tissue type	α4β7	CXCR3	CD38
Small intestinal Follicular Lymphoma	4/4	0/4	4/4
Follicular Lymphoma +	2/21	0/6	3/3
MALT lymphoma +	14/15	6/6	0/4
B-cell chronic lymphocytic leukemia +	0/6	2/3	nd
Ileum *	4/4	0/7	5/5
Tonsil *	0/2	0/3	2/2
Lymph node *	0/2	nd	nd

n/n indicates: number of positive cases / number tested cases; nd, not done
+ The α4β7 stainings include also previously reported cases. (11 FLs, 10 MALT lymphomas and 6 B-CLLs) 26.
* Indicated is the reactivity of the monoclonal antibody with GC B cells. The GCs of the ileum showed a low expression with the anti-α4β7 monoclonal antibody.

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Molecular Pathways in Follicular Lymphoma

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Molecular Pathways in Follicular Lymphoma

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Abstract

Follicular lymphoma (FL) is one of the most common B-cell non Hodgkin's lymphomas. The initiating genetic event found in ~90% of follicular lymphoma is the t(14;18), causing constitutive expression of the anti-apoptotic BCL-2 protein. The exact secondary alterations leading to full FL development are still poorly defined. In this review we address (i) the genetic pathways associated with tumorigenesis and progression of FL (ii) the role of micro-environmental factors with emphasis on B cell receptor ligands and (iii) lymphoma models in mice and what they teach us about lymphomagenesis in man.

Introduction

Follicular lymphoma (FL) is the most common low-grade B-cell non-Hodgkin's lymphoma (B-NHL) mainly affecting adults with a peak incidence between the fifth and sixth decade. The disease has a variable clinical course with a median survival of ~10 years (1). FL generally is systemic involving lymph nodes, spleen, Waldeyers' ring, bone marrow and blood. Secondary involvement of 'extranodal' sites such as the gastrointestinal tract, soft tissue and skin may occur at advanced stages. FL is occasionally found primarily at extranodal sites such as the skin, ocular adnexa, gastrointestinal tract and the female genital tract (2). In the WHO classification, primary cutaneous FL is denoted as a FL variant, although this entity generally lacks the characteristic t(14;18)(q32;q21) (1;3). Surprisingly, primary gastrointestinal FL, which clearly stands out in having a characteristic clinical behavior, harboring the t(14;18) involving *IgH/BCL-2* and with specific immunophenotypic features, is not considered as a separate entity in this classification (1;4-6).

The cytological and architectural features of FL are highly reminiscent of those of normal germinal centers (GC). The tumor cells resemble normal centroblasts and centrocytes, and proliferate in follicles in a network of non-malignant follicular dendritic cells (FDC) and T cells. The neoplastic follicles lack a typical mantle zone of small naïve B cells (1). The tumor cells express B cell markers such as CD19, CD20, CD22, the GC B cell markers BCL-6, CD38 and CD10 and membrane bound immunoglobulin (mIgM>mIgG>mIgA). Furthermore, FL cells express CD95 (Fas) and the co-stimulatory molecules CD86 and CD40, although usually weaker than in normal GC B cells (2). In contrast to normal centrocytes and centroblasts, ~90% of FL express the anti-apoptotic BCL-2 protein due to the t(14;18) (7;8). Although this translocation, occurring in the bone marrow during Ig gene rearrangement, is considered as the essential first genetic hit, the t(14;18) by itself is not sufficient for FL development. This is illustrated by the finding that *bcl-2* transgenic mice, in which *bcl-2* is controlled by Eμ, do not readily develop lymphoma (9-11). Moreover, in healthy human individuals, B cells containing a t(14;18) are normally present (12-14). Additional secondary (genetic) alterations are thus necessary for FL development.

A small fraction of FL (~5%) does not exhibit the classical t(14;18) but instead contains alterations affecting *BCL-6* at 3q27, including t(3;14)(q27;q32). This leads to deregulated expression of the transcriptional repressor BCL-6, normally required for GC formation (15-19). These FL most often display an exclusive centroblastic morphology, may be BCL-2$^-$ and are classified by the WHO as grade 3B (1).

Somatic immunoglobulin gene alterations and lymphomagenesis

Immunoglobulin (Ig) V(D)J recombination and somatic hypermutation (SHM) of *IgV* genes as well as class switch recombination (CSR) bear intrinsic risks for introduction of

non-physiological genomic alterations. V(D)J recombination is exerted during early B-cell development in the bone marrow where the Ig variable regions of both heavy (IgV_H) and light (IgV_L) chain are formed by rearrangement of germline-encoded Ig gene segments. At the recombination signal sequences (RSS) of the respective V, D and J gene segments, double stranded DNA breaks are introduced by the RAG enzymes after which the intervening DNA is excised. Next, the selected Ig gene segments are joined by the general DNA-repair machinery, specifically by non-homologous end-joining (20-22). There is now ample evidence that the V(D)J gene recombination machinery can be instrumental in the generation of chromosomal translocations. The best examples are the canonical translocations found in FL, t(14;18)(q32;q21) and in mantle zone B-cell lymphoma (MCL), t(11;14)(q13;q32) in which the respective *BCL-2* and *BCL-1* loci are juxtaposed to the RSS regions of germline J_H genes. By consequence, these translocated genes become regulated by the powerful *IgH* Eμ enhancer leading to the constitutive expression of the anti-apoptotic BCL-2 and the cell cycle progression regulator cyclin-D1, respectively (7;8;23). Recently, direct proof of involvement of the V(D)J recombination machinery in this process was provided, as it was shown that the DNA of the major breakpoint region in *BCL-2* often acquires an altered structure, making it susceptible for RAG-mediated cleavage (24).

The fact that most mature B-NHLs are of GC or post GC phenotype indicates that the GC, where vigorous proliferation is combined with extensive DNA modification, is a hazardous place. Somatic hypermutation (SHM) alters the affinity of the IgV_H and IgV_L chains for the antigen (Ag). Mutated subclones with the highest affinity for Ag will retain survival signals elicited by Ag and finally predominate the GC reaction. Subsequently, the Ag-selected B cells may undergo Ig class switch recombination (CSR). In this process, the switch (S) region sequence of IgM/IgD is recombined with one of the downstream S region sequences, 5' of each constant region gene (Cγ3, Cγ1, Cα1, Cγ2, Cγ4, Cε and Cα2). As a consequence, the rearranged V_H-D_H-J_H region is expressed as either an IgG, IgA or IgE antibody (Ab). Finally, the Ag-selected (either or not class switched) B cells will differentiate into either memory B cells or Ab-producing plasma cells (25-27). SHM and CSR essentially depend on the B-cell specific enzyme activation-induced cytidine deaminase (AID). As both processes are associated with the occurrence of single- and double- stranded DNA breaks it is presumed that they predispose to chromosomal translocations (28-33). Indeed, translocations involving *IgH* S region sequences, likely due to erroneous CSR have been found, e.g. *BCL-6* in FL and in diffuse large B cell lymphoma (DLBCL) (34;35), *C-MYC* in sporadic Burkitt's lymphoma (BL) and in DLBCL (36) and *Cyclin-D1*, *Cyclin-D3, FGFR3-MMSET* and *C-MAF* in multiple myeloma (37). Interestingly, a recent study on *IgH* S region breakpoints in t(3;14)(q27;q32) (*BCL-6/IgH*) demonstrated that in FL most *IgH* breakpoints involved Sγ whereas in DLBCL mostly Sμ regions are involved. This difference in molecular anatomy provokes

distinct control of BCL-6 expression: In FL, the Sγ breakpoint implies that BCL-6 expression is driven by an Iγ promoter whose activity requires CD40 and cytokine signaling. In DLBCL, the Sμ breakpoint leads to BCL-6 expression driven by the constitutively active intronic *IgH* enhancer (38). Translocations due to aberrant SHM include *BCL-6* in DLBCL and *C-MYC* in endemic BL, which are both positioned into rearranged and somatically mutated IgV_H or IgV_L regions (36). In addition, *BCL-6* translocations to various other non-Ig genes such as *RhoH/TTF* and *PIM-1* have also been found in DLBCL (39;40). These fusions of *BCL-6* are compatible with the fact that in ~30% of normal GC B cells the 5' portion of *BCL-6* is targeted by SHM and that *RhoH/TTF* and *PIM-1* are among the genes that are aberrantly targeted by SHM in a proportion of DLBCL (41-43). Table 1 provides an overview of the chromosomal translocations found in B-NHL entities and indicates the proposed underlying pathogenetic mechanisms. In addition to *RhoH/TTF* and *PIM-1* also *C-MYC* and *PAX-5* can be targets for aberrant SHM in DLBCL (41). Analyses of these aberrant mutations indicated that they indeed had all the features of SHM, i.e. clustering up to 1-2 kb downstream of the transcription initiation sites and the preferential targeting at the RGYW motifs. Apparently, in the course of DLBCL development, the tight control of AID targeting can become defective. In FL, SHM of the 5' untranslated region of *BCL-6* was found in ~40% of the cases (42;44) while aberrant SHM was not found in *PIM-1*, *C-MYC* and *PAX-5* and found rarely in *RhoH/TTF* (ref (41) and our unpublished results).

Generally, the oncogenic translocations in the *Ig* loci of B-NHL occur in non-productively rearranged *Ig* alleles thereby allowing Ig (BCR) expression in most B-NHL. Indeed, virtually all FLs express functional BCRs, containing somatically hypermutated IgV_H and IgV_L genes (45;46). In comparison to normal GC B cells, FLs contain significantly more mutations in the IgV_H genes, which is compatible with prolonged expansion in a GC-like environment. The average number of mutations in IgV_H of IgM expressing- and isotype-switched- FLs are 23.0 (7.8%) and 32.0 (10.9%), respectively (47;48).

Several investigators believe that, given their strong resemblance to normal GCs, FLs have retained essential functional properties of their non-neoplastic counter-parts as well, in particular the capacity to actively hypermutate their *IgV* genes. This idea was supported by the observation of so-called intraclonal IgV nucleotide variation (ICV) among tumor subclones. Significant ICV is indeed found in the vast majority of FLs (47). However, based on analyses of sequential biopsies of a total of 9 FLs, spanning significant periods, we questioned whether ICV is indeed the result of ongoing SHM (47;49-51). In only 2 out of these 9 FLs, we observed evidence compatible with ongoing SHM, i.e. ICV at both time points, accumulation of the total number of IgV_H mutations over time and sustained AID expression (Figure 1) (47;52). One FL showed no ICV at either time points whereas 6 FLs showed decreased ICV over time, without clear accumulation of the total number of IgV_H mutations. (Figure 1) (47;49-51). In

3 FLs, evidence was obtained for the selective outgrowth of minor subclones, that had been present already at the earliest timepoints (50;53;54). Importantly, AID mRNA expression levels did not correlate with the presence nor the level of ICV in FL nor in other B-NHL such as DLBCL and BL (52;55;56). Thus, as AID is essential for SHM, ICV alone cannot be taken as a measure for ongoing SHM.

Table 1. Chromosomal translocations of mature B-NHL

Lymphoma	Translocation	Involved genes	Proposed mechanism	% of cases (refs)
MCL	t(11;14)(q13;q32)	*Cyclin-D1/IgH*	V(D)J	~95% (23)
FL	t(14;18)(q32;q21)	*IgH/BCL-2*	V(D)J	~90% (57)
	t(3;14)(q27;q32)	*BCL-6/IgH*	CSR	~5% (17;18) [a]
MZBCL	t(11;18)(q21;q21)	*API-2/MALT*	?	(58-60) [b]
	t(1;14)(p22;q32)	*BCL-10/IgH*	CSR	~5% (58)
	t(14;18)(q32;q21)	*IgH/MALT*	CSR	(58) [c]
	t(3;14)(p14.1;q32)	*FOX-P1/IgH*	CSR	(61-65) [d]
DLBCL	t(14;18)(q32;q21)	*IgH/BCL-2*	V(D)J	15-30% (66) [e]
	t(3;14)(p14.1;q32)	*FOX-P1/IgH*	CSR	(61-65) [d]
	t(3;14)(q27;q32)	*BCL-6/IgH*	CSR	~35% (34;35)
	t(3;various)(q27)	*BCL-6/various*	SHM	~5% (34;35)
	t(8;14)(q24;q32)	*C-MYC/IgH*	CSR/SHM	~10% (67)
	t(8;22)(q24;q11)	*C-MYC/Igλ*	SHM	~5% (67)
BL	t(8;14)(q24;q32)	*C-MYC/IgH*	CSR/SHM	↓
	t(8;22)(q24;q11)	*C-MYC/Igλ*	SHM	BL together 100% (68;69)
	t(2;8)(p11;q24)	*C-MYC/Igκ*	SHM	↑
MM	t(11;14)(q13;q32)	*Cyclin-D1/IgH*	CSR	15-20% (37)
	t(6;14)(p21;q32)	*Cyclin-D3/IgH*	CSR	~5% (37)
	t(4;14)(p16;q32)	*(FGFR3-MMSET)/IgH*	CSR	~15% (37)
	t(14;16)(q32;q23)	*C-MAF/IgH*	CSR	5-10% (37)

Abbreviations: MCL, Mantle cell lymphoma; FL, Follicular lymphoma; MZBCL, Marginal zone B-cell lymphoma; DLBCL, Diffuse large B-cell lymphoma; BL, Burkitt's lymphoma; MM, Multiple myeloma [a] t(3;14) and t(3;various) is merely found in FL grade 3B with an exclusive centroblast-like morphology (17;18). [b] The frequency depends on the primary site of the MZBCL. t(11;18) is found in ~40% of pulmonary MZBCL and in ~25% of gastric MZBCL (58-60). [c] The frequency depends on the primary site of the MZBCL. t(14;18) is found in ~25% of ocular adnexa MZBCL, ~10% of cutaneous MZBCL and in ~10% of salivary gland MZBCL (58). [d] t(3;14) (*FOX-P1/IgH*) translocations have been identified in MZBCL and in DLBCL, mostly with extranodal presentation.
The frequency of t(3;14) is low and the distribution among the various primary lymphoma sites is momentarily not exactly clear (61-65). [e] t(14;18) is merely found in the GC B type of DLBCL (67).

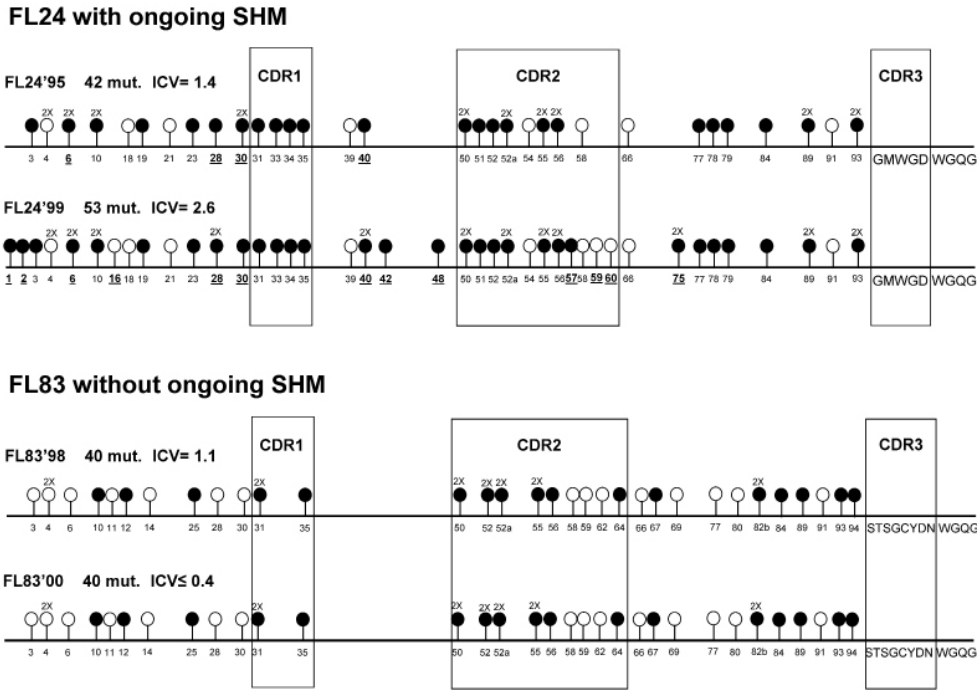

Figure 1. Serial biopsies of two follicular lymphomas, with and without ongoing IgV_H somatic hypermutation.

Schematic representation of IgV_H consensus sequences including somatic mutations, obtained from serial biopsies of FL24 and FL83. The lollipop shaped symbols indicate nucleotide differences as compared with the V3-23 germline *IgV_H* gene of FL24 and FL83, respectively. Replacement- and silent- mutations are indicated as closed and open circles, respectively, with codon numbering according to V-Base indicated underneath. The underlined codons in FL24 represents dissimilar mutations between FL24'95 and FL24'99, in total 15 mutations were different between the two time points of FL24. 2X, two mutations in the indicated codon. In FL24, the total number of IgV_H mutations as well as the intraclonal variation (ICV) increased over time, compatible with ongoing SHM. In contrast, in FL83 the total number of IgV_H mutations did not increase over time whereas the ICV even decreased over a 2 year period, which is compatible with clonal outgrowth and no ongoing SHM. In both biopsies of FL24, AID mRNA expression levels were comparable to those of normal GC B cells. In both biopsies of FL83 AID mRNA expression was not measurable (52). At least 7 individual IgV_H clones were sequenced of the two time points of both FLs. The ICV is calculated as the mean number of additional IgV_H mutations of the individual molecular IgV_H clones, as compared to the consensus IgV_H sequence of a given FL.

The role of the B-cell antigen receptor complex in follicular lymphoma development

During all phases of normal development, B cells are continuously selected based on signals transmitted by the BCR. Depending on the differentiation stage, BCR signaling has different outcomes. Immature B cells in the bone marrow will, upon strong BCR signals elicited by autoantigen undergo either IgL chain editing, functional inactivation through induction of anergy or be clonally deleted (70;71). In the mature B cell compartment however, Ag binding generally elicits strong stimulatory signals provoking extensive proliferation and

differentiation (27). In the GC, the rapidly expanding B cells are critically dependent on BCR signaling; cells that lose contact with Ag due to affinity-lowering IgV mutations, rapidly die by apoptosis (25-27). In addition, based on BCR ablation experiments in mice, it has become clear that the mere expression of an intact BCR generates autonomous survival signals, also designated as tonic BCR signaling (72;73). Inducible interference of the signaling capacity of the BCR associated Igα, in transgenic mice, showed that the BCR maintenance signal is transmitted through the Igα/Igβ heterodimer (74). Moreover, small interfering RNA (siRNA) specific for Igα and Igβ were recently used to inhibit BCR expression on murine and human B cell lymphoma cell lines which was accompanied by reduced cell proliferation/survival as well as by reduced phosphorylation of Syk and its downstream targets (75).

The IgV_H and IgV_L are each composed of four framework regions (FR1 – FR4), essential for the overall Ig structure, and three complementarity determining regions (CDR1 – CDR3), the actual contact sites for Ag. The IgV_H-CDR3, located at the V_H-D_H-J_H junction, is the most hypervariable region and considered of major importance for the antigenic specificity of an Ig. Based on size differences of D_H gene segments and due to the processes of nucleotide deletion as well as insertion of non-templated nucleotides during V(D)J rearrangement, the CDR3 region displays a 15-85 bp length variation among B cells. In (post) GC B cells, amino acid replacement mutations are preferentially found in the CDRs, thereby altering the affinity for the Ag. The FRs contains more silent mutations than would be expected on the basis of chance alone. This low replacement/silent (R/S) ratio in the FR is considered to be the result of selective forces in the GC to preserve the global structure of the expressed Ig. Interestingly, in accordance with these functional restrictions to the BCR, evolutionary forces created CDRs and FRs with codon compositions with intrinsic biases for replacement and silent mutations, respectively (76).

By statistical calculations, it was demonstrated that in ~90% of FLs significant counterselection for replacement mutations in the FRs of the IgV_H genes is exerted. Thus, despite high somatic mutation loads, the overall structure of the IgV_H and thus of the BCR is preserved in FL (47;76;77). For comparison, in ~40% of DLBCLs such counterselection for R mutations in FRs is not found. Accordingly, about half of DLBCL lack BCR protein expression, indicating that they are less dependent on BCR signals than FLs are (78).

Since the BCRs are structurally intact in FLs they may still bind Ag which is most likely present at the surface of non-neoplastic follicular dendritic cells (FDC) in the malignant follicles. However, immunohistochemical staining using 4 recombinant FL-derived soluble Igs did not reveal reactivity with any structures in the corresponding FL tissues nor did these antibodies bind auto-Ags such as IgG and nuclear-Ags (79). In a previous study by Dighiero et al., 8 out of 31 FL-derived Igs reacted in vitro with nuclear-Ags, IgG, actin and tubulin (80). As deduced from IgV_H/IgV_L structures of FLs, no clues were found concerning binding

of recurrent antigenic epitopes. The IgV_H gene repertoire of FL is comparable to that of normal B cells and amino acid sequence analyses of FL IgV_H-CDR3 did not reveal recurrent motifs (46;47;79;81). Moreover, the mean IgV_H-CDR3 amino acid sequence length of FL is comparable to that of normal naïve B cells (79;82). Since long IgV_H-CDR3 regions have been associated with auto- and poly- reactive antibodies, this finding is not in support of autoreactivity of FL BCRs (82). In contrast, B-cell chronic lymphocytic leukemia (B-CLL) and gastric- and salivary gland- mucosa-associated lymphoid tissue (MALT) lymphomas express clearly restricted, albeit different, IgV_H/IgV_L repertoires with recurrence of IgV_H-CDR3 motifs and proven auto-antigen specificity (79). In addition, B-CLL with unmutated IgV_H genes express on average longer IgV_H-CDR3 regions which is in accordance with their frequent auto- and poly- reactivity (79). Thus, in general FLs seem to recognize unique epitopes. In our opinion it is unlikely that FLs are still engaged by their specific Ags during the tumor stage. In view of the systemic spread, it is difficult to envisage how sufficient amounts of non-self Ags could become widely distributed. One exception may be the primary gastrointestinal (GI) FLs. These rare FLs harbor the t(14;18) involving *IgH/BCL-2* as determined by PCR, evolve in the gut mucosa, express the mucosal homing integrin $\alpha4\beta7$, typically express IgA and have a low tendency to spread systemically and by consequence have a good clinical prognosis (4-6;83). These features indicate that GI-FLs originate from local, antigen-responsive precursor cells and their sessile nature suggests continued dependence on Ags, e.g. originating from the gut lumen, also during the tumor stage (4).

It has been reported that in 55 of 79 FLs analyzed (79%) replacement mutations in IgV_H result in generation of amino acid motifs being potential N-glycosylation sites (84). This frequency is significantly higher than that of DLBCL (41%), mutated B-CLL (13%), multiple myeloma (8%) and normal (post) GC B cells (9%) (84). It has been proposed that creation of new N-glycosylation sites may provide growth advantage and are being selected for during FL development. Supra-physiological N-glycosylation may enhance Ag-independent BCR signaling (tonic signaling) or, alternatively enhance specific or non-specific interactions with autologous structures present in the micro-environment (84). In this respect it is of interest that a more rapid and sustained BCR mediated signaling has recently been demonstrated in FL B cells as compared to normal B cells (85).

Micro-environmental factors

Tumor B cells in FL proliferate in close contact with T cells, dendritic cells (DC), macrophages, follicular dendritic cells (FDC) and other stromal cells. These cells likely support FL proliferation by typical adhesion molecules and other stimulatory surface molecules as well as by production of growth factors/cytokines. Generally, FL cells express CD40, the costimulatory molecule CD86 and less often CD80. The ligands, CD28/CD152

(CTLA4) for CD80/CD86 and CD40L (CD154) for CD40, are expressed by intratumoral T cells (86;87). FDCs express ICAM1 and VCAM1, the counterstructures for LFA-1 (CD11a/CD18) and VLA-4 (CD49d/CD29) present on FL cells (88;89). Although FDCs may also present native Ag to the FL cells, immunohistochemical staining using 4 FL-derived soluble Igs did not reveal binding to any structures or cells in the corresponding lymphoma tissues (79). FDCs also provide stimulatory molecules such as IL-15, 8D6 (CD320), BAFF and hepatocyte growth factor (HGF), which may all have a role in normal GC B-cell expansion and may exert a similar function in FL (90-95). Production of TNF by B cells is critically important for the generation of fully developed FDCs out of resident stromal cells upon p55-TNF-R signaling (96-98). Moreover, sustained interaction of TNF and $LT\alpha_1\beta_2$ is important for the maintenance of mature FDCs networks (99;100). It has been shown that sorted FL B cells produce TNF, LTα and LTβ (101). The production of these molecules supposedly is crucial for the local generation of FDCs or FDC-like cells out of stroma at extranodal sites and the bone marrow.

Immunophenotypical characterization studies revealed that in 14/35 (40%) of FL the FDC phenotype was comparable to that of FDCs in normal GCs, i.e. with expression of CD23, CD21, CD35, CXCL13 (BLC), low affinity nerve growth factor receptor (LNGFR) and CNA.42. In the other 21 FLs, the FDCs displayed a more stromal phenotype expressing CXCL13 but with either partial 11/35 (31%) or complete 10/35 (29%) absense of the established FDC antigens CD23, CD21, CD35 and with variable expression of LNGFR and CNA.42 (102). The FL with 'full' FDC phenotype contained numerous intra-follicular T cells, similar to reactive GCs, whereas in FLs with a 'stromal' FDC phenotype a paucity of intrafollicular T-cells was observed (102). Thus, FL with 'stromal' type FDC may be less reliant on T-cells. Moreover, serial biopsies showed progressive loss of 'full' GC FDC phenotype in approximately half of the cases over time (102). Possibly, reduced TNF and/or $LT\alpha_1\beta_2$ production by tumor B cells may result in loss of 'full' GC FDC phenotype and increased trafficking of the tumor B cells within the lymph node and beyond (54).

Secondary gene alterations in follicular lymphoma

It is assumed that DNA remodeling processes that take place in the GC are instrumental in completing transformation of t(14;18)-carrying B cells to FL. Numerous genetic defects have been recorded but, since only a few secondary DNA alterations are known to occur in a substantial fraction of FL, scenarios for stepwise transformation are still ill-defined. Höglund et al.(103) identified, by classical cytogenetics on 336 $t(14;18)^+$ FL 5 recurrent chromosomal alterations, each occurring in at least 20% of the FL: -1p32-36 (20%), -6q11-27 (30%), +7 (28%), +12 (23%) and +X (24%). The mean number of imbalances per FL was ~6 with most FLs having 2 imbalances. Similar analyses by Mohamed et al.(104) on 52 $t(14;18)^+$ FLs

yielded compatible results: -1p32-36 (36%), -6q11-27(14%), +7 (27%), +12 (8%) and +X (27%). When accounting for alterations found in more than 5% of FLs, Höglund defined 28 genetic regions. Evaluation of these 28 recurrent alterations in individual FLs designated +1q, +7, +8, +12 and +der(18) as early imbalances and 2p-, 10p-, -15, 17p- and 17q- as late imbalances. Applying the "principal-components" analysis method, the authors identified +7, 6q- and +der(18) and likely +1q as the four major events occurring immediately after the t(14;18) (Figure 2) (103). Altogether, 4 karyotypic routes of FL development were proposed, of which the +7, 6q-, +(der)18 and +1q karyotypic routes accounted for 34%, 20%, 10% and 8% of the FLs, respectively (Figure 2). In an univariate analysis of 165 FL patients of which complete clinical information was available, 6 alterations (+X, 1p-, +1q, +12, 17p- and 17q-) were defined as significant negative predictors of overall survival (103). Inferior outcome of the +X group was confirmed in another study on 124 FLs analyzed by comparative genomic hybridization (105). Moreover, in this study –6q25-27 was identified as the strongest predictor of dismal prognosis, confirming an earlier study which reported that patients suffering from FLs with breaks at 6q23-26 had a significantly shorter survival time (105;106). Finally, -6q and +12 had already been correlated with aggressive disease in an older study (107).

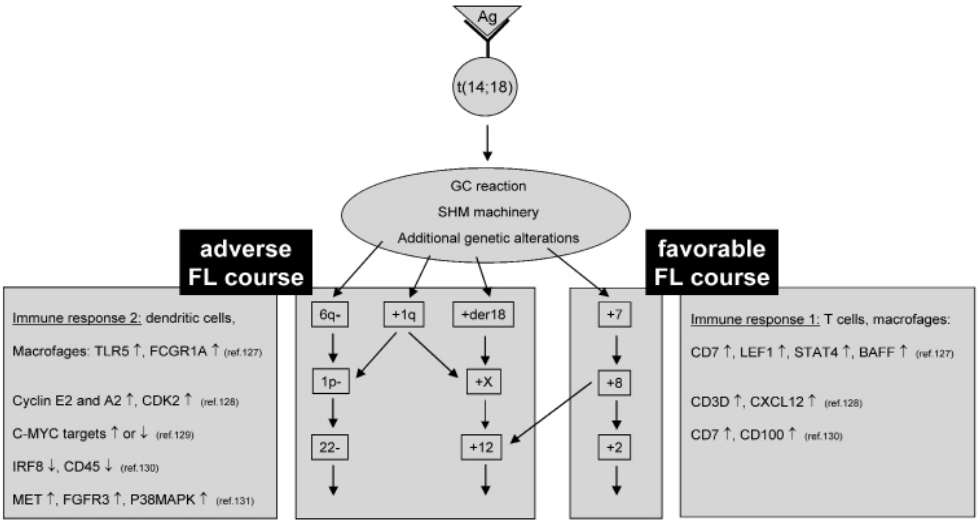

Figure 2. The genetic pathways of follicular lymphoma in relation to the clinical course.

The alterations affecting chromosome 1, i.e. -1p32-36, +1p11-q44 and unbalanced translocations of these regions [der(1)t(1;1)(p36;q11-23)] are among the most common secondary alterations in FL (108). Many candidate tumor suppressor genes map to 1p36, including *CDC2L1, TNFR2, ID3, PAX7, DAN, TP73, RUNX3* and *SKI*. However, of none

of these genes a causal relationship with FL development has yet been shown (109). The unbalanced t(1;1)(p36;q11-23) is observed in ~3% of FL and leads in most cases to deletion of *MEL1, TP73* and *SKI* at 1p36 and replacement by the 1q21 genes *MUC1, JTB, AFQ1* and, depending on the breakpoint, also of *BCL-9* and *IRTA1/2* (108). Interestingly, *MUC1* and *BCL-9* have also been implicated in *IgH* translocations in a DLBCL and in an acute lymphoblastic leukemia, respectively (110;111). Moreover, the *FCGR2B* gene (FcγR-IIB) also at 1q21 has been found in *IgL* translocations secondary to t(14;18), as well as in *IgH* translocations, in a small number of FLs (112;113). Deletions at 6q11-27 are frequently found in FL and are associated with an adverse prognosis (105-107). In two studies on the 6q11-27 deletions, a region of minimal deletion was identified at 6q16.3, harboring the *SIM1, RNAH, DJ* and *GRIK* genes (114;115).

Due to accumulation of genetic damage, FL may progressively transform into a lymphoma with a higher number of centroblasts, a less clear or absent follicular architecture and disappearance of FDC networks, eventually being indistinguishable from a DLBCL. Histological and clinical FL progression is associated with a variety of molecular alterations such as inactivation of *CDKN2A* ($p16^{INK4A}$, ARF) and *CDKN2B* ($p15^{INK4B}$) by chromosomal deletion, mutation and/or hypermethylation (116-118), *C-MYC* gene rearrangement (119) and mutation of the 5' untranslated regulatory region of *BCL-6* (120), of the translocated *BCL-2* gene (121) and of *TP53*, respectively (122-124). Each of these genetic events occur in a subset of transformed FL only. However, some of these events may affect similar regulatory pathways. For example, mutation and subsequent overexpression of the transcriptional repressor BCL-6 as well as deletion of ARF results in increased suppression of P53, which de facto has the same effect as *TP53* mutation (125).

Gene expression profiling in FL and FL evolution

Most gene expression studies on FLs were performed using RNA out of whole tumor tissue samples, thereby analyzing in addition to the FL cells significant numbers of stromal cells, T cells and other immune cells. This strategy a priori complicates comparison of the expression data derived from FLs with those found in normal, sorted GC B cells. In one study, purified FL cells from 6 patients with recurrent disease were compared with normal GC B cells using an array of 588 cDNAs (126). In these FLs, markedly enhanced expression levels were found for *SMAD1* (TGFβ signaling protein 1), the MAP kinase *MAP3K11* (MLK3), the cell cycle regulator *CDKN1A* ($p21^{Cip1}$), heat shock proteins *HSPB1* and *HSPF1* (Hsp27 and Hsp40), *TNF* and the transcription factors *ID2* and *JUN* (c-jun). Markedly decreased expression levels were encountered for the S100 protein family members *MRP8* and *MRP14*, also known as migration inhibitory factor-related proteins 8 and 14 (126).

Dave et al.(127) analyzed global gene expression of whole FL tissue samples of 191 patients

in relation to the clinical prognosis. The authors defined expression profiles, termed the immune-response-1 and -2 signatures, associated with long and short survival, respectively. Immune-response 1 implies expression of T-cell specific genes *CD7, CD8B1, LEF1, ITK* and *STAT4* and macrophage lineage genes *ACTN1* and *TNFSF13B* (BAFF). The immune-response 2 signature includes genes expressed by macrophages and/or dendritic cells, like *TLR5, FCGR1A* (FcγR-I), *SEPT10, LGMN* and *C3AR1* (complement 3a receptor 1). Cell sorting experiments confirmed that the immune-response signatures of the FL tissues mainly reflect expression levels of the various, non-neoplastic $CD19^-$ cell populations.

Several groups compared expression profiles of low grade FL (histological grades 1-2) with those of corresponding biopsies of grade 3 FL or FL that had transformed into a lymphoma with DLBCL morphology. Based on 12 of such paired FL samples, Glas et al.(128) extracted a set of 81 genes which showed optimal accuracy in classifying low-grade and high-grade FL disease. Expression of these 81 genes was also used in 58 single FL biopsies to assess clinical FL behavior. Aggressive FL disease was associated with upregulation of genes involved in cell cycle control such as *CCNE2* (cyclin E2), *CCNA2* (cyclin A2), *CDK2* (cyclin-dependent kinase 2) and genes reflecting increased metabolism and DNA synthesis. Genes associated with indolent FL disease were *CD3D* (CD3δ), derived of T cells, and *CXCL12* (SDF-1), expressed by stromal cells. Lossos et al.(129) studied 12 FLs with transformation and identified a set of 671 genes that exhibited at least a 3-fold variation in the biopsy pairs of three or more patients. According to these investigators, at least two distinct profiles can be discerned in association with FL progression: Five out of the 12 cases displayed enhanced expression of *C-MYC* and its target genes, while in 4 cases a decreased expression of *C-MYC* and its target genes was observed. De Vos et al.(130) studied 4 FLs with documented progression. Interestingly, of their top list of 36 upregulated and 66 downregulated genes upon transformation, 7 genes were also identified by Lossos et al.(129). Among others, this involved increased expression of *CDA* and *GAPD*, two genes reflecting levels of metabolism, and decreased expression of the transcription factor *IRF8* and of *PTPRC* (CD45). De Vos et al.(130) also noted down-regulation of different T cell markers upon transformation such as *CD7*, *FYB* (Fyn binding protein) and *SEMA4D* (CD100). Elenitoba-Johnson et al.(131) studied 11 FLs that transformed into DLBCL. Sixty-seven genes were found to be upregulated and 46 genes were significantly downregulated in the DLBCL. Markedly upregulated genes included the growth factor/cytokine receptors *MET* (the hepatocyte growth factor receptor), *FGFR3* (fibroblast growth factor receptor 3), *LTBR* (lymphotoxin β receptor) and *PDGFRB* (platelet-derived growth factor receptor β). In addition, *p38BMAPK* was upregulated in the DLBCL. This was confirmed immunohistochemically as phosphorylated p38BMAPK was detected in the nuclei of DLBCL and not in the majority of FLs nor in normal GC B cells.

Overall, the top lists of genes found, implicated in progression by the different investigators

show a low degree of concurrence. However, some findings from the different studies seem to be shared (Figure 2). Similar to Lossos et al.(129) also Glas et al.(128) observed that upon FL progression 4 C-MYC targets were upregulated, i.e. *LDHA, MTHFD1* (both reflecting increased metabolism), *NME1* (NM23-H1) and the cell cycle regulating *CKS2*. Dave et al.(127) divided FLs in two prognostic groups, merely based on the expression of markers from the non-malignant cells present in whole tissues. Interestingly, the good prognosis groups of Dave et al.(127), Glas et al.(128) as well as in the FLs before transformation of De Vos et al.(130) were characterized by high expression of T cell-related genes *CD7, CD8, CD3D, FYB* and *CD100* and of the FDC- and macrophage- derived *BAFF* (Figure2). These good prognosis groups may coincide with FL displaying follicles containing the immunohistochemically defined 'full' GC phenotype FDCs, which is also associated with the presence of numerous intra-follicular T cells (102). Of note, the good prognosis group of Dave et al.(127) (immune response 1) was not merely a reflection of the total number of T cells. No correlation was observed between pan-T-cell markers and survival, indicating that a specialized subset of T cells may be associated with improved survival. The bad prognosis group of Dave et al.(127) (immune response 2) was characterized by genes expressed by macrophages and/or dendritic cells. Two recent immunohistochemical studies corroborated these prognostic expression profiles. One study unveiled a correlation between higher number of FOX-P3$^+$ regulatory T cells and improved overall survival (132). Another study showed a correlation between higher number of CD68$^+$ macrophages and bad overall survival (133). In both studies no correlation was found between the number of CD4$^+$ T cells and survival (132;133). Interestingly, it was shown that the CD4$^+$ CD25$^+$ FOX-P3$^+$ regulatory T cells also expressed high levels of CD7, which was one of the T cell markers identified in the microarray studies (127;130;134). In addition, the number of FOX-P3$^+$ regulatory T cells decreased dramatically in 5 FLs that underwent transformation into DLBCLs (132). Regulatory T cells can either inhibit CD4$^+$ CD25$^-$ T cells, including GC T cells, which may support FL B cell proliferation/survival. Alternatively, they may also exert a direct inhibitory influence to FL B cells (132;132). Altogether, the data suggest that expression profiles of indolent FLs more closely resemble that of normal GCs. The gradual accumulation of genetic damage may yield tumor cell populations with diminished dependence on cell cell interactions. This may affect the microenvironment (e.g. loss of 'full' GC FDC phenotype and FOX-P3$^+$ regulatory T cells) which in turn favors outgrowth of autonomously proliferating subclones.

Mouse models of lymphomagenesis

Many mouse models have been developed that shed light on various oncogenetic and tumor suppressor pathways involved in lymphomagenesis. Although some of these models nicely mimic development of some human lymphoma entities, a convincing FL model is still not

available. Transgenic mice in which bcl-2 was driven by the *IgH* enhancer (Eμ) primarily developed follicular hyperplasia but not lymphoma (10;11). After one year, approximately 15% of the mice eventually developed large B cell lymphomas but no FLs. About half of these lymphomas contained *c-myc* rearrangements, indicating that additional genetic alterations are necessary for cellular transformation (9). Most likely, in man such secondary alterations in t(14;18)$^{+}$ B cells are acquired during the GC reaction and relate to the Ig diversification processes. B cells acquiring such DNA damage have a higher chance to escape from apoptosis due to the constitutive expression of BCL-2, normally being absent during the GC phase. Recently, an alternative *bcl-2* transgenic mouse model was presented in which bcl-2 was driven under control of the pan-hematopoeitic Vav-P promotor. In these mice mature isotype-switched lymphomas developed, ~45% of these lymphomas were cytologically reminiscent of FL but still lacked the typical follicular architecture (135).

Double Eμ-*bcl-2/myc* transgenic mice generated lymphomas much faster than both single Eμ-*bcl-2* and single Eμ-*myc* transgenic mice. The lymphomas in double Eμ-*bcl-2/myc* transgenic mice consist most often of mIgM^{-} lymphoblastic cells in which c-myc initiates robust proliferation, most likely the pro-apoptotic properties of c-myc are inhibited by bcl-2 (136). In the lymphomas of single Eμ-*myc* mice, the c-myc induced p53-dependent apoptosis pathway appeared frequently disturbed by loss of function of p53 or Arf or by elevated Mdm2 levels (137). Thus, a prominent second hit in the Eμ-*myc* model is the counteraction of the pro-apoptotic capacities of c-myc. This can be achieved either by inhibition of the Arf-Mdm2-p53 pro-apoptotic tumor suppressor pathway or by overexpression of the anti-apoptotic bcl-2 protein. Forced deletion of Arf or p53, as expected, accelerated lymphomagenesis in the Eμ-*myc* mice (137;138). Similarly, the *bmi-1* polycomb gene, which also strongly collaborates in c-myc induced lymphomagenesis, was shown to downregulate Arf (139). Eμ-*myc* mice that are deficient for the pro-apoptotic molecules Bim or Bax also showed accelerated generation of pre-B and mature B-cell lymphomas. In the lymphomas of most Bim deficient mice, the Arf-Mdm2-p53 pathway appeared unaffected, indicating that Bim deficiency is an effective alternative to loss of p53 function (140;141). Finally, *pim-1* was identified by random proviral tagging, as a strong collaborator of c-myc induced lymphomagenesis, which was confirmed in double Eμ-*pim-1/myc* transgenic mice (142;143). The mechanism by which pim-1 exerts its action is as yet incompletely understood but a role in anti-apoptosis and cell cycle progression has been claimed (144). Replacement of pim-1 by pim-2 had the same outcome in this model (145). Figure 3 provides an overview of the Eμ-*myc* mice models. The importance of c-myc in lymphomagenesis was further demonstrated in mice double-deficient for p53 and DNA damage repair factors such as Ku80, XRCC4, DNA ligase IV or DNA-PKcs. The pro- and pre- B cell lymphomas that evolve in these mice generally harbor spontaneous *IgH/myc* translocations (146).

It is now clear that the type of lymphoma induced by deregulated c-myc expression depends on the exact genomic localization of the rearranged c-myc. In the transgenic Eμ-*myc* models mentioned, most of the lymphomas phenotypically resemble ($mIgM^-$) pre B cells with lymphoblastic morphology. In models in which c-myc was driven from the human germline $V_H(D)J_H$-Cμ-Cδ locus in a "head to tail" orientation or from the Igλ locus in a "head to head" orientation, $sIgM/IgD^+$ immature B or transitional B-cell type lymphomas developed (147;148). Yet another outcome is seen in mice in which *c-myc* was inserted "head to head" into the *Igh* locus just 5' of Eμ, thereby mimicking both the human t(8;14)(q24;q32) of BL and the mouse T(12;15) of pristane induced plasmacytomas. After 21 months, much later than in all other c-myc mice models, 68% of these mice suffered from lymphomas, i.e. ~50% Burkitt-like lymphoma (BL-L), ~30% DLBCL and ~20% plasmacytoma. The BL-L/DLBCL were IgM/IgD^+ $B220^+$ $CD19^+$ $CD5^-$ and the plasmacytomas expressed CD138 (syndecan-1). Some lymphomas expressed class-switched Ig and the BL-L/DLBCL expressed bcl-6, suggestive for GC experience. However, the IgV_H genes of these tumors appeared to be unmutated. (149;150). Similar to the other Eμ-*myc* induced lymphomas, in 12 of 26 BL-L the Arf-Mdm2-p53 pathway was deregulated (149). Of note, in human BL the ARF-MDM2-P53 pathway is frequently disturbed due to deletions of ARF, elevated MDM2 levels or P53 mutations (151-153).

The transcriptional repressor BCL-6 is rearranged in ~40% of human DLBCLs and in ~5% of FLs (Table 1) (34;35). Transgenic mice were developed in which *bcl-6* was positioned "head to head" into the *Igh* locus under the control of the IgH Iμ promotor, thereby mimicking the human t(3;14)(q27;q32). The mice showed an increased number of GCs already before immunization and, after 6 months, ~40% of the mice harbored poly or oligoclonal B cell expansions. At 15-20 months, 50% developed mature IgM/IgD^+CD43^- B-cell lymphomas histologically reminiscent of human DLBCL and predominantly of splenic origin. Most lymphomas (82%) contained somatically mutated IgV_H genes, compatible with GC passage. The vast majority of the DLBCL contained clonal complex non-random cytogenetic abnormalities, indicating that, a single oncogenic hit had not been sufficient for lymphomagenesis. Expression of c-myc was heterogeneous, indicating that c-myc is not a dominant second hit in this model (154).

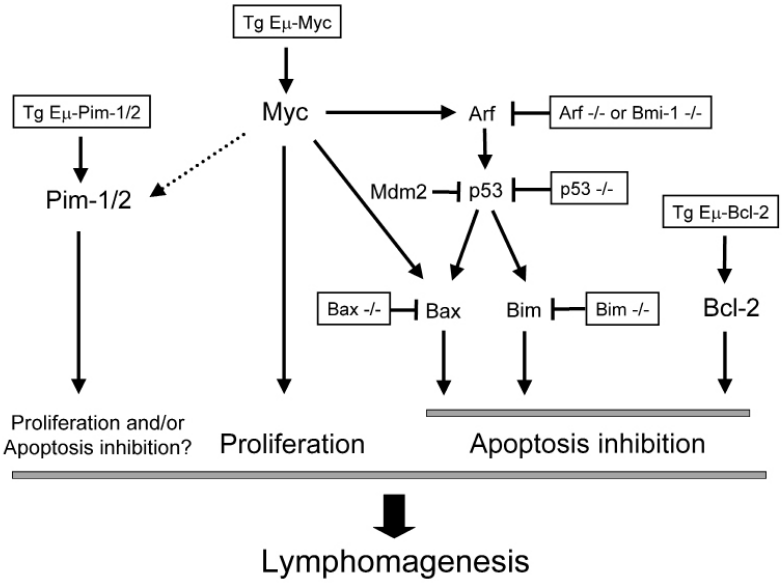

Figure 3. Inhibition of c-Myc induced apoptosis pathways accelerates lymphomagenesis in transgenic Eμ-myc mice. Additional molecular alterations introduced in Eμ-Myc mice are indicated in boxes; (Tg) transgenic or (-/-) knockout.

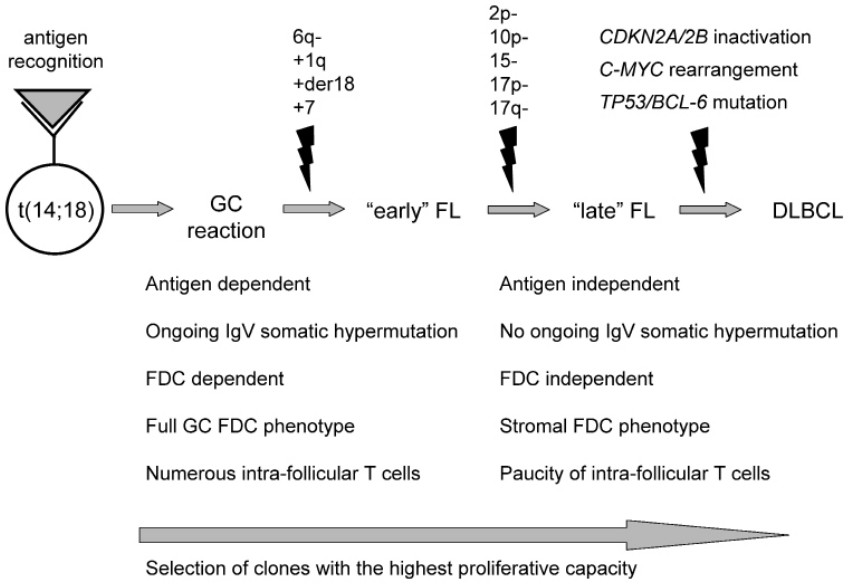

Figure 4. The pathogenesis of follicular lymphoma.

Summary

FL originate from B cells that acquired a t(14;18) during abnormal Ig gene rearrangement in the bone marrow. T(14;18)$^+$ B cells evolve at low frequencies in healthy individuals, which has no consequences unless these cells happen to meet Ag and become involved in a T-cell dependent GC reaction (Figure 4). Here, the t(14;18)$^+$ B cells obtain growth advantage over normal B cells since constitutive BCL-2 expression (i) interferes with strict selection processes normally favoring only offspring with highest Ig affinity for the Ag and (ii) prevents apoptosis of those cells that acquired DNA damage, most likely as a byproduct SHM and CSR. Accumulation of genetic alterations ultimately leads to formation of FL, a tumor that in earliest stages not only morphologically resembles normal GCs but still depends on Ag for its survival while hypermutating its *IgV* genes. FLs most likely arise randomly out of the pool of mature circulating t(14;18)$^+$ B cells, do not posses auto- nor poly-reactivity like some other lymphomas do, but recognize non-recurrent foreign Ags. According to this scenario, Ag will sooner or later become limited and finally be lost, favoring the outgrowth of Ag-independent tumor clones. Nevertheless, the long-term preservation of an intact Ig in virtually all FLs suggests a role for tonic BCR signaling in FL growth. During the ongoing transformation process, FLs become less dependent on, and competent in, maintaining the follicular micro-environment. In particular, reduced TNF and/or $LT\alpha_1\beta_2$ production by the tumor cells may be instrumental in the gradual transition of a 'full' GC FDC phenotype to a 'stromal' FDC phenotype which, concurrent with paucity of intrafollicular T-cells, culminates in the disappearance of follicular networks. At the latest stages, the lymphomas, consisting of autonomous blastoid cells primarily competing on basis of their proliferative capacity, become morphologically indistinguishable from DLBCL. A significant fraction of the DNA alterations found at this stage converge on the p53 and C-MYC pathways.

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Differential Noxa/Mcl-1 balance in peripheral versus lymph node chronic lymphocytic leukemia cells correlates with survival capacity

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Differential Noxa/Mcl-1 balance in peripheral versus lymph node chronic lymphocytic leukemia cells correlates with survival capacity

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Abstract

The gradual accumulation of chronic lymphocytic leukemia (B-CLL) cells is presumed to derive from proliferation centers in lymph nodes and bone marrow. To what extent these cells possess the purported anti-apoptotic phenotype of peripheral B-CLL cells is unknown. Recently, we have described that in B-CLL samples from peripheral blood, aberrant apoptosis gene expression was not limited to protective changes but also included increased levels of pro-apoptotic BH3-only member Noxa. Here, we compare apoptosis gene profiles from peripheral blood B-CLL (n=15) with lymph node B-CLL (>90% $CD5^+$/$CD19^+$/$CD23^+$ lymphocytes with $Ki67^+$ centers; n=9). Apart from expected differences in Survivin and Bcl-xL, a prominent distinction with peripheral B-CLL cells was the decreased averaged level of Noxa in lymph nodes. Mcl-1 protein expression showed a reverse trend. Noxa expression could also be reduced *in vitro* by CD40 stimulation of peripheral blood B-CLL. Direct manipulation of Noxa protein levels was achieved by proteasome inhibition in B-CLL and via RNAi in model cell lines. In each instance, cell viability was directly linked with Noxa levels. These data indicate that suppression of Noxa in the lymph node environment contributes to the persistence of B-CLL at these sites and suggest that therapeutic targeting of Noxa might be beneficial.

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by a progressive accumulation of monoclonal $CD5^+$ $CD23^+$ mature B cells in the secondary lymphoid tissues, bone marrow, and blood[1]. Previously, it was assumed that B-CLL is associated with a defective regulation of programmed cell death (apoptosis), rather than uncontrolled cell proliferation[2]. Indeed, high expression of the anti-apoptotic proteins Bcl-2 and Mcl-1 has been associated with rapid disease progression and a poor response to chemotherapy[3;4]. Paradoxically, investigation of virtually all direct apoptosis regulators known at present revealed that, in addition to these anti-apoptotic alterations, the pro-apoptotic proteins Noxa and Bmf are also abundantly expressed in B-CLL[5;6]. How the elevated expression of these pro-apoptotic proteins is associated with the reputed increased life span of the B-CLL cells is currently unknown.The vast majority of the circulating B-CLL cells is arrested in G0/G1 phase of the cell cycle[7], which has contributed to the view that B-CLL is an indolent disease. However, isotopic labeling of leukemic cells *in vivo* revealed that a substantial fraction of the B-CLL cells does proliferate[8]. It seems logical to assume that the generation of new cells takes place in so called proliferation centers frequently found in lymph nodes and bone marrow of B-CLL patients. This is supported by the numerous $Ki67^+$ and $Survivin^+$ cells present in these structures[1;9]. The microenvironment not only plays an essential role in the induction of proliferation but presumably also in the suppression of apoptosis. *In vitro* experiments revealed that various cell types can support the survival of B-CLL cells. Apart from follicular dendritic cells (FDC), bone marrow stromal cells, IL-6 producing endothelial cells, VCAM-1 and SDF-producing nurse-like cells, $CD4^+$ T cells can also aid in providing a microenvironment where B-CLL cells can survive and proliferate[10-14]. The importance of the microenvironment for the survival of B-CLL cells is also shown by the finding that despite the relentless accumulation of the B-CLL cells *in vivo*, culturing the leukemic cells *in vitro* results in spontaneous apoptosis[15;16]. *In vitro* culture of B-CLL cells in the presence of CD40L rescues the cells from spontaneous and drug-induced apoptosis, suggesting that such co-stimulatory signals play a role in the survival of B-CLL cells *in vivo* and even in the response to treatment[9;17-19].

To date, B-CLL is an incurable disease. Although multi-agent treatment can result in a profound peripheral lymphocyte depletion, the B-CLL cells in the bone marrow and/or lymph nodes are less effectively targeted[20]. Persistence of B-CLL cells in the bone marrow is associated with an increased risk of relapse[21]. Therefore, more molecular data about the B-CLL cells in the lymphoid tissues and bone marrow are necessary, preferably coupled with assessment of efficacy of therapeutics towards B-CLL residing in those niches. We here initiated such an effort by comparing a large panel of apoptosis regulators in circulating B-CLL cells and B-CLL cells residing in lymph nodes. Although the expression of most apoptosis regulators was remarkably comparable, a prominent difference was the expression of the BH3-only

protein Noxa. Furthermore, we demonstrate that CD40 engagement of peripheral B-CLL cells can largely reproduce the altered apoptosis profile found in lymph node B-CLL cells. Finally, we show that *in vitro* manipulation of Noxa expression has a significant and direct effect on B-CLL cell survival. Together, these data provide a new link between the anti-apoptotic microenvironment in the lymph nodes and suppression of Noxa, which suggests that drugs that increase Noxa levels, such as proteasome inhibitors[22-25], may be of therapeutic benefit in B-CLL.

Material en methods

Patient material and cell lines.

Patient material was obtained after routine diagnostic or follow-up procedures at the departments of Hematology and Pathology of the Academic Medical Center Amsterdam. All patients were diagnosed according to the WHO classification system[1]. Lymph node (LN) material diffusely infiltrated by B-CLL cells was freshly frozen in liquid nitrogen directly after surgical removal. Immuno-histochemical analysis (see below) of these lymph nodes revealed that more than 90% of the tissue consisted of tumor cells. Peripheral blood (PB) mononuclear cells (PBMC) of B-CLL patients were obtained after Ficoll density centrifugation (Pharmacia Biotech, Roosendaal, the Netherlands). PBMCs from B-CLL patients contained >75% $CD5^+$, $CD19^+$ cells as assessed by flow cytometry and were stored in liquid nitrogen as cell suspensions in 10% DMSO (Merck, Darmstadt, Germany) in heat-inactivated FCS (Invitrogen, Breda, The Netherlands). Clone FSA of the Burkitt's lymphoma cell line Ramos with enhanced response to CD95 has been described previously[24]. Cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM; Invitrogen), supplemented with 10% (v/v) heat-inactivated FCS (ICN Biomedicals GmbH, Meckenheim, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin and 5 mM L-glutamine (Invitrogen).This study was conducted in accordance with the ethical standards in our institutional medical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, revised in 1983.

RNA isolation and reverse transcription-multiplex ligation-dependent probe amplification assay (RT-MLPA).

Total RNA was isolated using the Nucleospin RNA isolation kit (Macherey-nagel, Düren, Germany). RT-MLPA procedure was performed as described previously[5;25]. Briefly, 100 ng total RNA was reverse transcribed using a gene-specific probe mix. The resulting cDNA was annealed overnight at 60°C to the MLPA probes. Annealed oligonucleotides were covalently linked by Ligase-65 at 54°C (MRC, Amsterdam, The Netherlands). Ligation products were

amplified by polymerase chain reaction (PCR; 33 cycles, 30 seconds at 95°C, 30 seconds at 60°C and 1 minute at 72°C) using one unlabelled and one 6-carboxy-fluorescein (FAM)-labeled primer (10 pM). PCR products were run on an ABI 3100 capillary sequencer in the presence of 1pM ROX 500 size standard (Applied biosystems, Warrington, UK). Results were analyzed using the programs Genescan analysis and Genotyper (Applied Biosystems). Category tables containing the area for each assigned peak (scored in arbitrary units) were compiled in Genotyper and exported for further analysis with Microsoft Excel spreadsheet software. Data were normalised by setting the sum of all signals at 100%, and expressing individual peaks relative to the 100% value.

Immunohistochemistry.

Monoclonal antibodies specific for CD5 (clone 4C7 Lab vision, Neomarkers, Fremont, CA), CD3 (clone SP7), CD23 (clone 1B12), Bcl-6 (clone PG-B67), were used on formalin-fixed paraffin-embedded lymph node specimens. When necessary, antigen retrieval was achieved using a TRIS-EDTA buffer pH 9.2. Antibody detection was performed with the Powervision$^+$ system (ImmunoVision Technologies, Daly City, CA) which was succeeded, for the single antibody staining, by peroxidase visualization with 3,3'-diaminobenzidine (DAB) (Sigma), 0.03% H_2O_2 in Tris-HCl pH 7.6. Finally, the sections were counterstained with haematoxylin, dehydrated and mounted in pertex. For the CD3/Ki67 double stainings the Ki67 MIB-1 clone (Dako, Glostrup, Denmark) was used, for the CD20/Ki67 double staining the Ki67 SP6 clone (Neomarkers), and the L26 clone from Dako. After antibody detection with the Powervision$^+$ system (ImmunoVision) the Liquid permanent red kit (Dako) was used, followed by peroxidase visualization with DAB (Sigma). Finally the slides were counterstained with haematoxylin and mounted in Vectamount.

Flow cytometry.

Purified B-CLL cells were incubated FITC- or PE-conjugated mAbs directed against CD5 (Sanquin), CD19 (Sanquin) and CD3 (Becton and Dickinson, San Jose, CA) and analyzed by flow cytometry with the CellQuest program on a FACS Calibur (Becton and Dickinson).

In vitro CD40 stimulation.

B-CLL samples were enriched to >95% purity from PBMCs via negative depletion as described previously[26]. In brief, T cells, monocytes and granulocytes were depleted using anti-CD3, anti-CD14 and anti-CD16 immunomagnetic beads on a magnetic particle concentrator (Dynal A.S. Oslo, Norway). The B-CLL cells were stimulated for three days in culture-treated 24-wells plates (Costar, Corning NY, USA). Each well contained 5 x 10^6 B-CLL cells and 1.5 x 10^5 irradiated (30 Gy) CD40L-transfected or untransfected fibroblast (NIH3T3).

Retroviral constructs and transduction.

To knock-down Noxa, pRetro-super was used, which contains the polymerase III H1-RNA promoter (pol3) for transcription of the siRNA probe and the phosphoglycerin kinase (*pgk*)1 promoter driving GFP expression[27]. The siRNA sequences were: N7 5'GAAGGTGCATTCATGGTG3' and N8 5'GTAATTATTGACACATTTC3'. The retroviral plasmids were transfected into the helper virus amhotropic producer cell line Phoenix with Fugen-6 (Roche Diagnostics , Almere, The Netherlands). For transduction, Ramos-FSA cells were exposed overnight to viral supernatant (containing vector GFP-only or one of the two Noxa RNAi-targeting sequences) on retronectin-coated (Takara Shuzo, Otso, Japan) 24-well plates. GFP-positive cells were sorted using a FACS-Aria (BD Biosciences) cell sorter to >90% purity for further experiments.

Analysis of apoptosis.

PB B-CLL cells were stimulated at a concentration of $5x10^6$ cells/ml with 20 nM bortezomib (Janssen-Cilag, Tilburg, The Netherlands) for four hours. The cells were washed twice with IMDM (when indicated) and incubated at given time points with FITC-labeled Annexin-V (IQ products, Groningen, The Netherlands) for 20 minutes. Prior to analyses, PI was added (final concentration 5 µg/ml). Viable cells were defined by Annexin V^-/PI^- staining. Ramos. FSA clones expressing either control-GFP or Noxa-RNAi (N7 or N8) were stimulated at a concentration of $5x10^5$ cells/ml with 30 nM bortezomib for 24 hours, harvested and incubated with 200 nM MitoTracker Orange (Molecular Probes, Leiden, The Netherlands) for 30 minutes at 37°C, washed and double-stained with APC-labeled Annexin-V (IQ products). Fludarabine, staurosporine and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-human Fas10 (agonistic antibody to the CD95 receptor) were a kind gift from Prof. Dr. L. Aarden (Sanquin, Amsterdam, The Netherlands).

Western Blotting.

Western Blotting was done as described previously[5]. Protein samples were separated by 13% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting. Blots were probed with the following antisera: polyclonal Mcl-1 (cat. no 554103, Pharmingen, BD Biosciences), monoclonal anti-Noxa (clone 114C307.1, Imgenex, San Diego, CA, USA), monoclonal anti-Bim (clone 14A8, Chemicon, Temecula, CA, USA) and antiserum to β-actin (clone I-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Protein bands were quantified using high resolution (1200 dpi) scanned images of exposed films and AIDA image analyzer software v3.5 (Raytest Gmbh; Straubenhardt, Germany). Exposed films were only considered when software indicated that bands were not overexposed. In each sample, background corrected intensity of Mcl-1 or Noxa bands were normalized for actin.

Statistical analyses.

The Mann Whitney U test was used to analyze if differences in gene expression between the PB and LN B-CLL were statistically significant. P-values < 0.01 were considered statistically significant. Densitometric scans of western blots and MLPA analyses of CD40-triggered CLL cells were analyzed with Student's T-test. P-values <0.05 were considered statistically significant.

Results

Patients characteristics and immunohistochemistry

Table 1. Patient and B-CLL sample characteristics.

Patient	location	age	Rai-stage	lympho* %	$CD5^+CD19^+$ %	CD19 %	CD3 %	IgV_H mutations
B-CLL24	LN	52		> 90	nd	nd	nd	-
B-CLL27	LN	64	1	> 90	76	76	10	nd
B-CLL28	LN	80	4	> 90	64	93	8	-
B-CLL30	LN	46	3	> 90	37#	72	26	-
B-CLL33	LN	76	2	> 90	nd	nd	nd	-
B-CLL35	LN	67	2	> 90	nd	nd	nd	-
B-CLL36	LN	59	4	> 90	93	93	5	-
B-CLL37	PB	62	1	nd	nd	nd	nd	+
B-CLL38	PB	62	0	98	97	97	2	+
B-CLL39	PB	72	3	nd	94	98	3	+
B-CLL40	PB	70	3	93	48#	98	1	-
B-CLL41	PB	nd	4	94	99	99	1	-
B-CLL42	PB	48	0	69	76	76	17	-
B-CLL43	PB	54	2	91	96	96	4	-
B-CLL44	PB	55	2	86	91	91	0	+
B-CLL45	PB	63	4	82	98	98	2	+
B-CLL46	PB	nd	nd	82	nd	nd	nd	nd
B-CLL47	PB	54	2	83	nd	nd	7	-
B-CLL48	PB	59	2	75	89	89	4	+
B-CLL50	PB	69	0	nd	99	99	3	+
B-CLL167$	PB	70	0	62	84	86	11	+
B-CLL183$	PB	78	0	47	94	95	4	+
B-CLL226$	PB	64	1	70	98	98	1	-
B-CLL261$	PB	77	4	32	92	92	4	+
B-CLL25	PB	68	2	nd	nd	78	13	-
	LN		2	> 90	82	83	20	-
B-CLL31	PB	72	2	nd	81	81	8	-
	LN		2	> 90	72	81	12	-

LN, lymph node; PB, peripheral blood; nd not done. IgV_H mutations + if ≥2% of the IgV_H gene was mutated.
* % lymphocytes was investigated in the lymph node samples by immunohistochemistry and in the peripheral blood samples by FACS analysis. # These samples displayed low CD5 staining and therefore the combined CD5/CD19 gate yielded low values. $These samples were used only for western blot analyses.

Lymph nodes from 9 B-CLL patients and peripheral blood samples from 15 B-CLL patients were included in the study. From 2 patients (B-CLL25 and B-CLL31) both lymph node (LN) tissue and a peripheral blood (PB) sample was available (Table 1). All B-CLL expressed CD5, CD23 and CD19/CD20. The B-CLL cells of the patients with LN involvement expressed unmutated immunoglobulin heavy chain (IgV_H) genes. Of the peripheral blood B-CLL, 10 expressed mutated IgV_H genes and 8 unmutated IgV_H genes (Table 1).

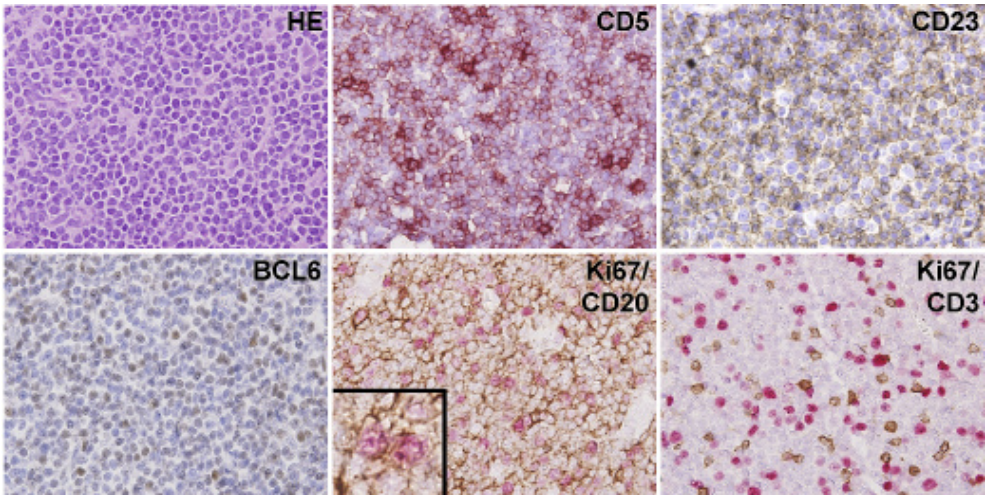

Figure 1: Histology of lymph node infiltrated by B-CLL cells .
Ubiquitously present B-CLL cells were positive for CD23 and CD5. Scattered $CD3^+$ T cells were present throughout the LN. The absence of clusters of $BCl\text{-}6^+$ cells excluded the presence of germinal center remnants in the LNs. Ki67/CD20 and Ki67/CD3 double staining indicate that all cycling $Ki67^+$ cells (pink) were of $CD20^+$ (brown) origin - see also inset -, while the $CD3^+$ T cells were predominantly Ki67 negative. Magnification 40x.

In the peripheral blood samples, at least 75% of the leucocytes were lymphocytes. Due to low levels of CD5 expression, the standard FACS gating yielded low percentage of $CD5/CD19^+$ cells in some patients; Patient 30 had in addition low numbers of circulating cells and was first diagnosed as small lymphocytic leukemia (SLL). Immunohistochemistry demonstrated that >90% of the LNs consisted of leukemic lymphocytes. $Ki67^+$ cells were present in all LNs, either diffusely or in proliferation centers. These cells were of B cell origin, as demonstrated by double staining which showed that all $Ki67^+$ cells were also $CD20^+$. In contrast, the scattered $CD3^+$ T cells were generally negative for Ki67. Absence of clusters of $Bcl\text{-}6^+$ or $CD21^+$ (data not shown) cells excluded the presence of germinal center remnants in these LNs (Figure 1).

Profiling of apoptosis genes in peripheral blood and LN samples of B-CLL.
The relative expression 34 known apoptosis regulators was investigated by RT-MLPA[5;25;28] in PB samples of 13 B-CLL patients, LN samples of 7 B-CLL patients (Figure 2A) and paired

PB and LN samples of 2 B-CLL patients (Figure 2B and data not shown).

The relative expression of the majority of the investigated genes was remarkably comparable between the PB and LN samples. We have described previously that, compared to normal tonsillar B cell fractions, in PB B-CLL several anti- and pro-apoptosis genes (e.g. Flip, Bcl-2, Noxa and Bmf) are aberrantly expressed[5], and this was also found in LN samples of B-CLL. Interestingly, 3 genes were differentially expressed in PB B-CLL cells as compared to LN samples (Figure 2C).

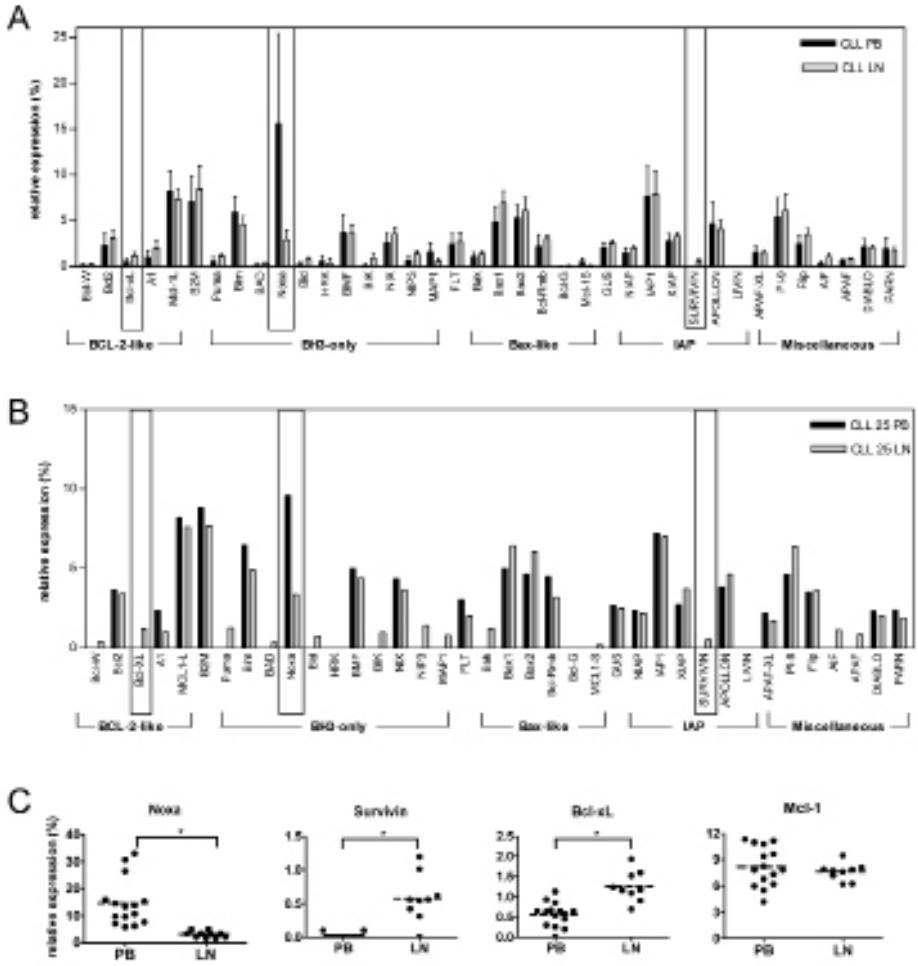

Figure 2: Apoptosis gene expression profile of B-CLL cells in peripheral blood and lymph nodes.
(A) Relative expression of 34 apoptosis regulators was investigated in 15 PB B-CLL (black bars) and 9 LN B-CLL (grey bars). Results of individual apoptosis regulatory genes are shown as expression relative to the total signal in the sample, with standard deviation. Non-apoptosis genes included as housekeeping genes are β2-microglobulin (B2M), Ferritin Light chain (FLT), β-glucoronidase (GUS), and poly(A)-specific ribonuclease (PARN). (B) RT-MLPA data from PB and LN sample of B-CLL-25. (C)The expression of Noxa, Survivin, Bcl-xL and Mcl-1 in individual patients are depicted as dots. Asterix (*) indicates statistical significance ($P<0.001$) of differences in gene expression between PB and LN B-CLL.

In agreement with previous reports, the IAP family member Survivin was not expressed in any of the PB B-CLL samples whereas it was clearly expressed in LN B-CLL[5;9] (P=0.0005). Also, the anti-apoptotic Bcl-2-family member Bcl-xL was more abundantly expressed in the LN samples (P=0.0003).

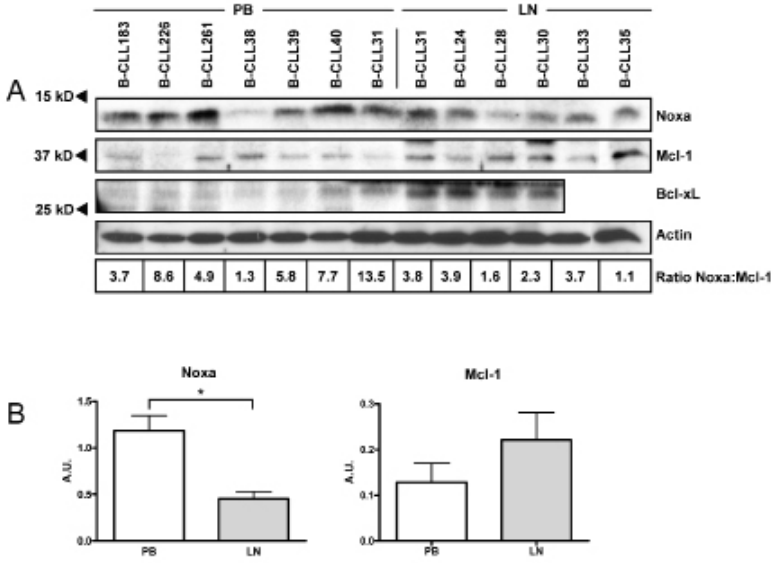

Figure 3: Comparison of Noxa, Mcl-1 and Bcl-xL protein in PB vs. LN B-CLL. Protein lysates of 7 PB samples and 6 LN samples were subjected to western blot analyses. **(A)** Blots were stained with antibodies directed against Noxa, Mcl-1 or Bcl-xL, and reprobed with an antibody against β-actin as a loading control. In case of Bcl-xL, aspecific staining at the upper cutting edge of the blot is visible and precluded analysis of the rightmost two samples. Densitometric scanning was performed, and Noxa/Mcl-1 ratios, corrected for actin levels, are indicated below the samples. The averaged Noxa/Mcl-1 ratio was significantly different between PB and LN (P= 0.011). **(B)** Averaged Noxa/actin and Mcl-1/actin ratios are separately plotted for PB and LN samples. Unpaired T-test showed that Noxa ratios were statistically significant (P=0.0026), and Mcl-1 ratios showed a non-significant trend.

The most striking difference in expression was observed for the BH3-only member Noxa. As found previously, this apoptogenic gene is abundantly expressed in PB B-CLL cells[5], but its expression was clearly lower in LN B-CLL cells (averaged relative expression of 15.6±9.8 in PB B-CLL versus 3.0±1.1 in LN B-CLL; P<0.0001 **(**Figure 2C). Of note, a difference in Noxa expression was also observed between the paired PB- and LN samples of an individual patient (relative expression 9.6 in the PB sample versus 3.4 in the LN sample) (Figure 2B). Western blot analyses confirmed that the differences in Bcl-xL and Noxa mRNA expression were also present at the protein level. The B-CLL LN samples generally expressed lower levels of Noxa than the PB B-CLL samples, and the reverse was observed for Bcl-xL (Figure 3).

Comparison of the RT-MLPA data with the Western blot data revealed a clear correlation between the levels of Noxa mRNA and Noxa protein. As reported previously, no differences were observed in expression of these apoptosis genes among IgV_H-mutated versus unmutated cases[5]. Since Noxa can selectively interact with the anti-apoptotic protein Mcl-1 and this may influence the degradation of Mcl-1[29], we investigated the expression of this Bcl-2 family member in PB B-CLL and LN B-CLL. Although RT-MLPA showed no difference in mRNA expression (Figure 2C), Western blot analyses revealed that in most LN B-CLL, where Noxa levels were low, Mcl-1 was slightly elevated. Furthermore, the PB samples that expressed higher levels of Noxa showed a decreased expression of Mcl-1 (Figure 3). This is further illustrated by a paired PB/LN protein sample, where in fact Noxa expression was almost equal, but in this case Mcl-1 protein levels were clearly higher in the LN compartment. Densitometric scanning of Noxa and Mcl-1 protein levels (ratio indicated below individual lanes in Fig 3A) also showed divergence between LN and PB, of which the averaged differences in Noxa levels were statistically significant (Figure 3B). In summary, the majority of the apoptotic regulators is expressed equally in PB - and LN B-CLL, but a novel and prominent distinction in Noxa level was found.

Noxa expression is modulated by CD40 engagement in B-CLL cells.

In the LNs the $CD40^+$ B-CLL cells are in close contact with T cells that may express CD40L[2] (Figure 1). To investigate the effect of this interaction on the expression of the apoptotic regulators, PB B-CLL samples (n = 11) were co-cultured for 1-5 days with CD40L-transfected or untransfected 3T3 fibroblasts (Figure 4). As reported previously, CD40 stimulation resulted in increased expression of Bcl-xL, A1/Bfl-1, Bid and Survivin[9;17;18]. Interestingly, in accordance with our findings in the LN B-CLL cells, CD40L-stimulated PB B-CLL cells also showed a diminished expression of Noxa (Figure 4A). The effects were observed after one day of CD40 stimulation and RT-MLPA performed at day three and day five showed that the expression of the apoptosis regulators did not alter significantly after that (Figure 4B). It should be noted that the levels of Noxa mRNA as compared to t=0 also decreased after culture on the control 3T3 cells ($p=0.019$). The reason for this is not known, however a stronger decline in Noxa levels was consistently observed after CD40 ligation ($p=0.004$), and the difference between control and CD40-treated cells was statistically significant ($p=0.016$). These differences were further investigated at the protein level for three patients (Figure 4C). Concordant with RT-MLPA analyses, Noxa levels decreased after 3 days culture in presence of CD40L-expressing cells, and Bcl-xL levels increased. Mcl-1 protein levels also clearly increased upon CD40-triggering, although this was not observed via RT-MLPA. So, similar to findings in LN samples (Figure 3), Mcl-1 levels were apparently under post-transcriptional control.

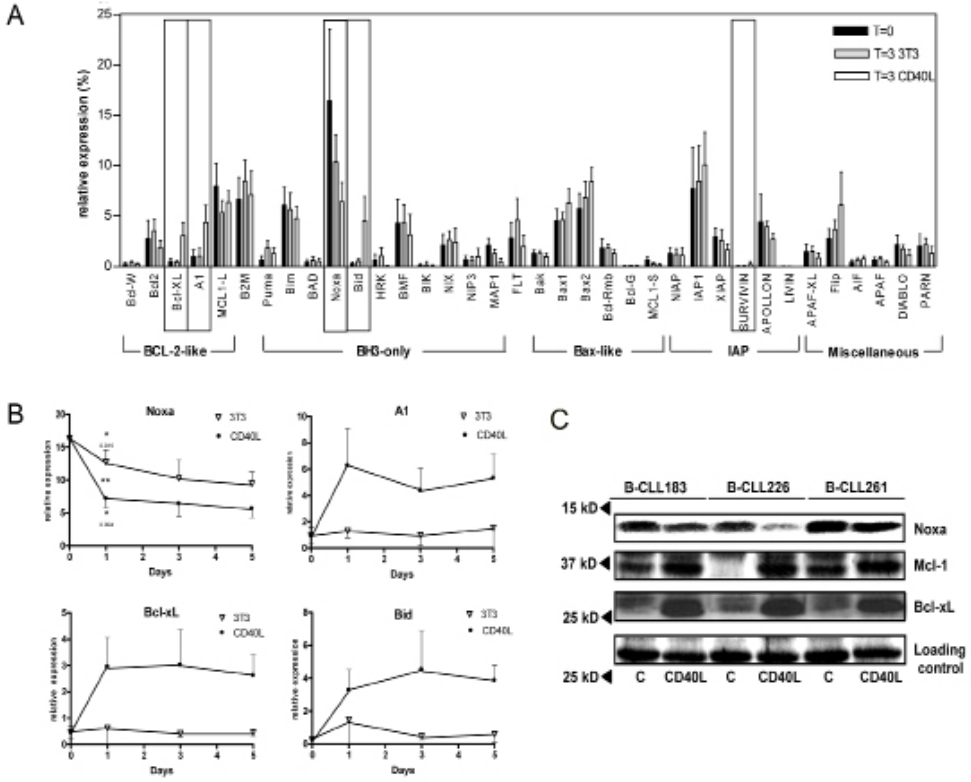

Figure 4: CD40 stimulation of peripheral blood B-CLL results in an apoptosis gene expression profile similar to lymph node B-CLL. **(A)** Apoptosis gene expression profile was investigated by RT-MLPA in PB samples of 11 freshly isolated B-CLL patients without culturing (black bars) and after three days of culturing on either 3T3 cells (grey bars) or CD40L-transfected 3T3 cells (white bars). Data plus standard deviation are presented as in Figure 2. **(B)** The expression of Bcl-xL, Bfl-1/A1, Bid and Noxa are shown at day 1, 3 and 5 of culturing on 3T3 cells (white triangles) or CD40L-transfected 3T3 cells (black dots)**.** Statistical analysis of day 0 vs. day 1 samples showed that in all cases the CD40L treated values were significantly different ($P<0.01$). In case of Noxa, there was also a small but significant decrease for the 3T3 control cells ($P=0.019$), and a more pronounced effect for CD40L treated cells ($P=0.004$; difference between 3T3 and CD40L-treated cells $P=0.0159$, indicated by **). **(C)** Western blot of t=0 samples in comparison of CD40L treated cells at day 3 for Noxa, Mcl-1 and Bcl-xL showed that Noxa protein levels decrease while Mcl-1 and Bcl-xL increase. For B-CLL sample 226 the Mcl-1 levels at t=0 were in fact undetectable (see also Figure 3)

A prominent distinction between CD40-stimulated B-CLL and LN B-CLL was found for expression of the apoptogenic BH3-only protein Bid. In contrast to LN B-CLL, CD40L-stimulated B-CLL showed a strong and continuous induction of Bid (Figure 4B). Thus, the altered gene expression in LNs can be mimicked largely but not entirely by *in vitro* CD40 engagement of B-CLL cells.

Bortezomib-induced Noxa upregulation causes apoptosis of PB B-CLL cells.

To establish a functional relationship between Noxa expression levels and apoptosis sensitivity of B-CLL cells, we made use of recent findings that proteasome inhibitors rapidly and specifically upregulate Noxa[22;23;30]. To reduce a widespread impact of proteasome inhibition on protein levels and transcription dependent processes[31], PB B-CLL cells were transiently exposed to bortezomib for 4 hours.The reversible proteasome inhibitor was then either washed away or incubation was continued. As expected, a pulse of bortezomib treatment already caused a rise in Noxa protein, and this was sufficient to impair survival of B-CLL cells (Figure 5).

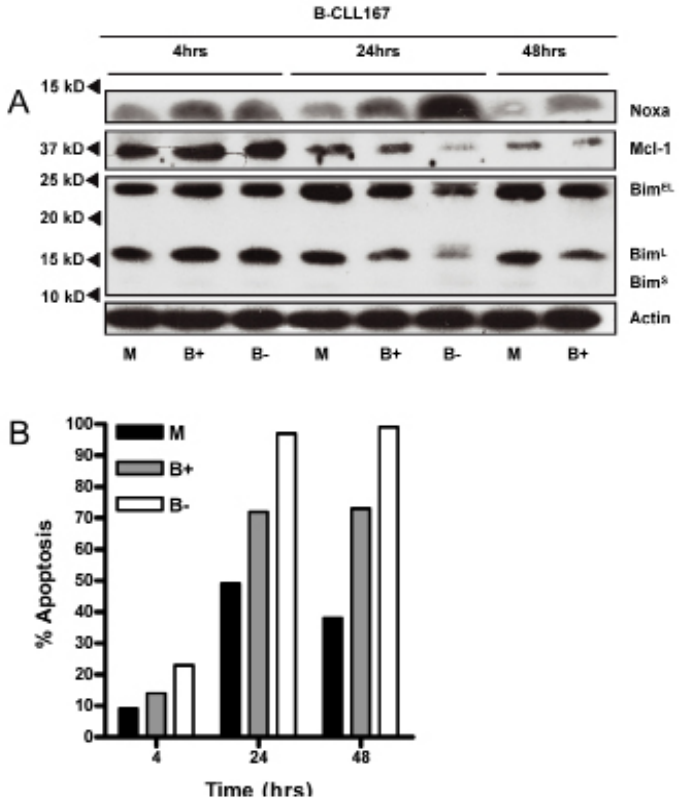

Figure 5: Noxa upregulation via transient treatment with bortezomib impacts CLL survival

Freshly isolated peripheral blood B-CLL cells were treated for 4 hrs with 20 nM of the proteasome inhibitor bortezomib. Cells were then washed and cultured in fresh medium, or incubation was continued. **(A)** At the indicated timepoints, cell lysates were prepared and probed for expression of Noxa, Mcl-1, Bim and Actin protein by western blot. Indicated below the lanes: untreated (M), bortezomib washed away after 4 hrs (B+), and bortezomib without washing (B-). The decrease in Mcl-1 levels in bortezomib treated cells at 24 and 48 hrs could be inhibited by the pan-caspase inhibitor z-VAD (data not shown). Due to massive cell death after 48 hrs in the presence of bortezomib, these lysates did not yield sufficient protein for analysis. **(B)** Apoptosis of cells was determined via AnnexinV staining. Spontaneous apoptosis in medium was approximately 50%, which was increased by bortezomib treatment. Results are representative for 3 separate experiments

Continuous exposure to bortezomib resulted in a massive increase in Noxa levels that was accompanied by almost 100% cell death at 48 hrs. Over the course of this experiment, Mcl-1 protein levels first increased(4 hr timepoint), most likely due to proteasome inhibition, and then declined when cells went into apoptosis. This decline could be prevented by blocking caspase activity with z-VAD (data not shown), and is thus in accord with reports that Mcl-1 is a caspase substrate[32;33]. Next, we investigated whether the level of Bim, another pro-apoptotic binding partner of Mcl-1[34], was also subject to change upon bortezomib treatment, and might thereby trigger apoptosis. However, Bim levels were unaffected, both as detected by RT-MLPA (data not shown), and by western blotting (Figure 5A). Thus, pharmacological manipulation of the levels of Noxa protein in B-CLL cells appeared to be directly related to viability in an *in vitro* setting.

Noxa-deficient cells exhibit resistance to bortezomib-induced cell death.

Apoptosis regulatory genes as detected via RT-MLPA were not affected during the short-term bortezomib treatment in the previous experiments (data not shown). Yet, it can not be excluded that other genes and proteins besides Noxa that might impact survival were affected by bortezomib. Therefore, to investigate a direct role for Noxa in bortezomib-induced apoptosis, we employed a model system. Ramos B cells (clone FSA)[24] were transduced with distinct retroviral constructs encoding Noxa siRNAs (N7 or N8) or control-GFP. GFP-positive cells were sorted and western blot analysis revealed a suppression of Noxa-levels to approximately 50-75% compared to the control-GFP (Figure 6A). Both Ramos FSA cell lines expressing Noxa RNAi exhibited a significant resistance to bortezomib-induced apoptosis compared to the mock-transduced cells (Figure 6B). The partial resistance to proteasome inhibitor-mediated apoptosis matched the partial knock-down of Noxa protein. Of note, also in Noxa RNAi cells, bortezomib treatment caused a rapid increase in Noxa protein (data not shown), thus explaining that apoptosis still occurred at higher concentration of the drug. These data are in good agreement with effects of Noxa knock-down in other celltypes (melanoma, mantle cell lymphoma and T cell leukemia)[22;23;30]. In addition, we obtained similar findings with another protease inhibitor (MG132; data not shown). In contrast, no effect of Noxa protein reduction was observed on apoptosis triggered via other pathways such as fludarabine or staurosporin treatment, or triggering of the CD95 receptor (Fig 6C). In conclusion, these data demonstrate that decreased expression of Noxa has a direct and specific impact on the susceptibility to apoptosis induced by proteasome inhibitors. Conversely, the death-inducing effect of proteasome inhibition observed in B-CLL cells may therefore rely predominantly on shifts in Noxa expression.

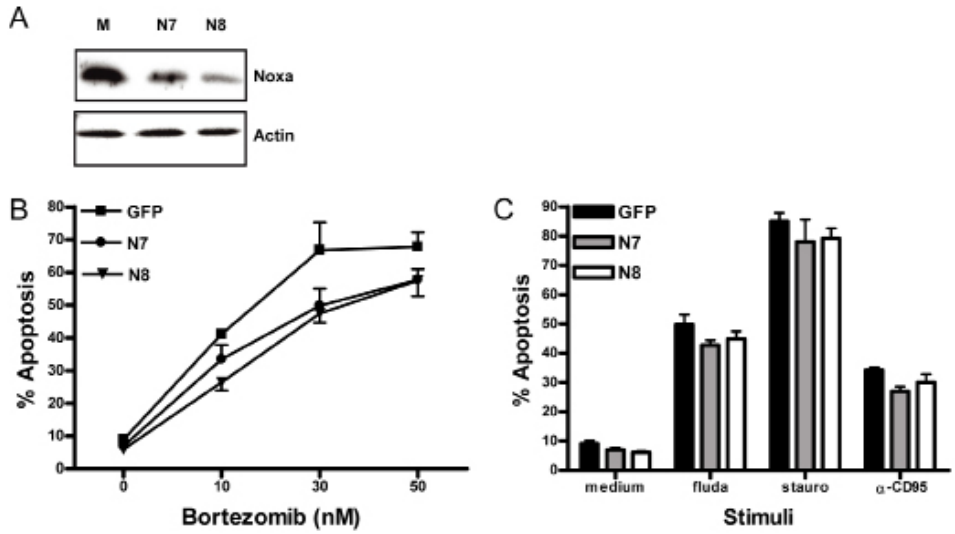

Figure 6: Noxa reduction via RNAi specifically prevents apoptosis induction by proteasome inhibitors
Ramos Burkitt lymphoma cells were retrovirally transduced with two RNAi constructs targeting Noxa (N7 or N8), or GFP control. **A)** Western blot demonstrating reduced Noxa expression in Ramos-N7 and -N8. Equal protein loading is shown by reprobing for **β-**Actin. **B)** Mock, N7 and N8 transduced Ramos FSA cells were cultured 24 hours in the presence of indicated concentration of bortezomib. Viability was assessed by AnnexinV/mitotracker staining and FACS analysis. Data represent mean ± SD from three independent experiments. **C)** Cells were incubated for 24 hrs in medium containing 100 μM fludarabine (fluda), 0.25 μM staurosporine (stauro), or 5 μg/ml α-CD95, and analysed as in B.

Discussion

There is increasing awareness that the B-CLL population in lymphoid proliferation centers differs fundamentally from the well studied fraction in PB and that this distinction may have clinical relevance[8;35]. Here, we present a first direct comparison of these two populations, focusing on the expression of 34 apoptosis regulatory genes. Apart from expected differences in proliferation-related genes (Survivin and Ki67) and anti-apoptotic Bcl-xL, we observed a prominent divergence in the expression of pro-apoptotic Noxa. Previously we described that, compared to non-malignant tonsil or peripheral B-cell fractions, B-CLL cells in the periphery display significantly increased levels of this BH3-only member of the Bcl-2 family, in a p53-independent manner[5]. The high levels of Noxa and another BH3-only member Bmf[6], contrasted with the purported anti-apoptotic phenotype of B-CLL cells[2] but remained functionally unexplained. Our new findings show that the Noxa level is considerably lower in LN CLL and that this is linked with survival capacity. Therefore, targeting Noxa expression or function could be of clinical benefit, also in p53 deficient cases.

In vitro CD40 stimulation of PB B-CLL cells resulted in a clear reduction of Noxa expression. Within the LN microenvironment, CD40 stimulation is most likely delivered by $CD40L^+$ T

cells. Several groups have investigated the effects of *in vitro* CD40 engagement in B-CLL cells[9;14;17;18;36-39] but an effect on Noxa expression was not yet reported. It is well known that CD40-stimulated B-CLL cells are more resistant to spontaneous or drug-induced apoptosis. This is most probably due to the induction the transcription factor nuclear factor κB (NFκB) and as a consequence, the expression of various anti-apoptotic genes, such as Bcl-xL, cIAP2, A20 and Flip[36]. Previously, Noxa was proposed to be a p53-response gene[40], but in B-CLL cells, Noxa is apparently not under control of p53, as illustrated by the clearly divergent expression of Puma and Noxa upon p53 stimuli[5;41]**.** Later, various transcription factors were proposed to regulate Noxa such as E2F1, p73 and hypoxia inducible factor HIF-1α[42-45]. Therefore at present it is difficult to definitely assign a specific signaling route that mediates Noxa expression. Very recently though, it was reported that HIF-1α is overexpressed in peripheral B-CLL cells[45], which may constitute a potential link to the increased Noxa levels in B-CLL.

Although CD40 stimulation of PB B-CLL cells resulted in a similar apoptosis gene expression profile to LN B-CLL, several genes deviated from this profile, most prominently Bid, as reported previously[17], but also A1/Bfl-1. This indicates that in the LN, B-CLL cells also receive other stimuli than CD40. Indeed, apart from $CD4^+$ T cells expressing CD40L, other cell types can support survival of B-CLL cells. *In vitro* culture with an FDC cell line or dendritic cells protects B-CLL cells from spontaneous apoptosis[14;46]. FDC-mediated survival was reported to depend on the expression of the Bcl-2 family member Mcl-1[14] and *in vitro* experiments revealed that Mcl-1 levels decline in B-CLL cells undergoing apoptosis[3;47]. Interestingly, recent data indicate that Mcl-1 is a preferred binding partner of Noxa[34], and we have indeed observed association of Mcl-1 with Noxa in primary B-CLL samples (D. Hallaert; manuscript in preparation). Furthermore, in 293T cells, Noxa has been described to mediate the degradation of Mcl-1[29]. If this mechanism also holds true for B-CLL cells, it may explain the increase in Mcl-1 protein we observed in LN B-CLL, which was not accompanied by an increase in Mcl-1 mRNA. Accordingly, augmented Mcl-1 protein levels are a consequence of the downregulation of Noxa in the LNs, rather than a environmental effect on Mcl-1 RNA expression. In addition, *in vitro* triggering of CD40 on B-CLL cells also influenced Mcl-1 levels in a post-transcriptional fashion (Figure 4). Thus, the differences in protein levels observed by us for Noxa, Mcl-1 and Bcl-xL levels in the B-CLL LN environment, correspond with current models based on the differential interaction potential of these Bcl-2 family members[29;34], and support the anti-apoptosis phenotype of B-CLL cells at this location compared to PB. In addition, spontaneous apoptosis *in vitro* of B-CLL cells may be connected with the high levels of Noxa which eventually saturate the short-lived Mcl-1 protein[29;48].

Two separate experimental approaches supported a direct role for Noxa in survival capacity of B-CLL cells. First, we used the recently discovered rapid and direct effect of bortezomib

on Noxa levels[22;23;30] to demonstrate that short term bortezomib exposure also quickly induced Noxa protein in B-CLL cells, with a corresponding decrease in viability (Figure 5).

Although the levels of Bim did not change upon bortezomib treatment, a role for Bim during the actual triggering phase of apoptosis cannot be excluded. In model systems, Bim is capable of actively triggering Bax activation, while Noxa functions in as 'sensitiser' [49;50]. Secondly, a complementary experiment was performed in a model system where only Noxa levels were modified via RNAi. Here, a clear inhibitory effect of Noxa reduction towards apoptosis mediated by proteasome inhibition was observed, while other apoptosis pathways were unaffected (Figure 6).

Taken together, our data support a model where the viability of the malignant B-CLL clone within the LNs and possibly also the bone marrow, corresponds with low levels of Noxa and an upregulation of Bcl-xL and Mcl-1. In addition to these anti-apoptotic gene expression alterations, the B-CLL cells also receive proliferative stimuli as indicated by the Ki-67$^+$ and Survivin$^+$ cells. When the B-CLL cells enter the circulation these stimuli are lost, Noxa is upregulated, and Bcl-xL, Mcl-1 and Survivin are downregulated. As a result, the B-CLL cells may become prone to apoptosis, which can however still be prevented by the continuous high expression of Bcl-2. To what extent circulating B-CLL are actually undergoing apoptosis is difficult to detect directly. Freshly isolated CLL cells are mostly non-apoptotic but undergo rapid 'spontaneous' apoptosis *in vitro*, and recent calculations point to appreciable *in vivo* death rates[8]. Together, this suggests that apoptotic B-CLL cells are rapidly cleared from circulation *in vivo*. It is generally assumed that in the LNs and bone marrow the B-CLL cells are relatively protected against therapeutic drugs[20]. The circulating B-CLL cells that are already prone to apoptosis are more easily targeted, but the residual B-CLL cells in the LN/bone marrow will eventually lead to a relapse. Currently, there is much interest in application of novel, p53-independent drugs to treat B-CLL[51-53]. Our data provide new insight into the regulation of the apoptotic behavior of B-CLL cells, and also afford new clues for therapeutic intervention by targeting Noxa expression.

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8

General discussion

General discussion

AID and lymphoma initiation

In the Western world lymphomas account for 6% of all cancers and have an incidence of around 20 per 100.000 [1]. Usually, the number of T cells exceed that of the B cells in the human body, nevertheless >95% of the lymphomas are of B-cell origin [2;3]. In the past years, it has become clear that the formation and revision of the BCR that is essential for the humoral immune response also endangers the genomic integrity of the developing B cell. During early B-cell development in the bone marrow, VDJ-rearrangement results in double-stranded DNA breaks that can be resolved aberrantly, giving rise to translocations involving the *Ig* gene and *BCL2* or *cyclin* D1 [3-6]. Most of the B-NHL have a GC- or post-GC phenotype, indicating that also the genetic alterations that occur during the GC reaction in combination with the rigorous proliferation play a role in lymphomagenesis. Indeed, DSB have also been shown to occur during SHM and CSR [7;8]. Furthermore, sequence analysis showed that the breakpoints of many translocations that are characteristic for B-NHL, are found within rearranged and hypermutated IgV_H genes or switch regions [2;4;5;9].

It is hypothesized that AID, via the introduction of aberrantly targeted SHM (ASHM) in proto-oncogenes, plays a role in the formation of DSB and thus chromosomal translocations. Indeed, in part of the DLBCL, ASHM has been described in *BCL6*, *C-MYC*, *PAX5*, *PIM1*, *RHO-1* [10] and the region in which these mutations occur overlap with the major breakpoint regions of the translocations in which these genes can also be involved. Furthermore, AID transgenic mice developed T-cell lymphomas (but not B cell lymphomas) with extensively mutated *C-MYC* and *PIM1* [11]. *In vitro* experiments showed that AID can target a number of non-*Ig* genes in E.coli, yeast, fibroblasts and B-cell lines [12-14]. In gastric epithelial cells also the proto-oncogene *TP53* may be target of ASHM [15]. In fact, the group of Schatz showed that in mice GC B cells, AID triggers mutations in a great variety of genes[16]. Furthermore, in IL-6 transgenic mice that are prone to develop plasmacytomas, AID is required for the occurrence of IgV_H/*C-MYC* translocations [17]. Other mice experiments showed that IL-4 and LPS-stimulated B-cells developed IgV_H/*C-MYC* translocations only in the presence of AID [18]. Finally, IμHABCL6 transgenic mice carry AID-dependent ASHM in *PIM1* and IL4- plus LPS-induced IgV_H/*C-MYC* translocations. Interestingly, these mice develop significantly less GC -derived lymphomas in the absence of AID [19].

Taken together, at least in mice, AID is essential for the introduction of ASHM in proto-oncogenes, which may alter their functional and/or expression, and also may cause chromosomal translocations that plays a role in lymphoma initiation.

Somatic hypermutation in B-cell non Hodgkin's lymphomas

Consistent with their GC or post-GC phenotype and gene expression pattern, the majority of B-NHL carries SHM in their IgV_H genes (Table 1). It has been suggested that some of the B-NHL, like FL, DLBCL, BL and MALT lymphomas have retained their capacity to actively hypermutate their IgV_H genes [2;20;21]. This is mainly based on the presence of intraclonal IgV_H nucleotide variation (ICV) among tumor subclones. In chapter two we show that, in contrast to the pre-GC- lymphomas (MCL and B-ALL), and post-GC lymphomas (B-CLL and MM), AID is highly expressed in part of the GC lymphomas, like FL, BL and DLBCL. Importantly, immunohistochemistry showed a clear correlation between the mRNA expression and protein expression in these lymphomas (unpublished data). Western blot analysis and micro-array analysis performed by other groups confirmed AID expression in part of the FL and DLBCL [22-24]. We (and others) did not find significant AID expression in MALT lymphomas and MCL. Other studies did report AID expression in MALT lymphomas however, these expression levels were clearly lower than in DLBCL and could not be confirmed at the protein level [25]. Non-quantitative RT-PCR showed AID expression in MCL irrespective of their IgV_H mutational status [26]. However, Guikema *et al.* showed that none of their described MCL reached AID expression levels comparable to that of purified GC-B cells [27]. Together, these data indicate that significant AID expression is confined to a subset of B-NHL with a GC-phenotype.

In chapter six, we describe IgV_H sequence analysis of serial biopsies of FL and show that in only 2 out of 9 investigated FL there is an accumulation of SHM, an increase in ICV and significant AID expression. Since the great majority of the FL show ICV, these findings show us that in contrast to the general believe, the SHM machinery is active in only a subset of FL, and that the presence of ICV is not necessarily a reflection of ongoing SHM [28;29]

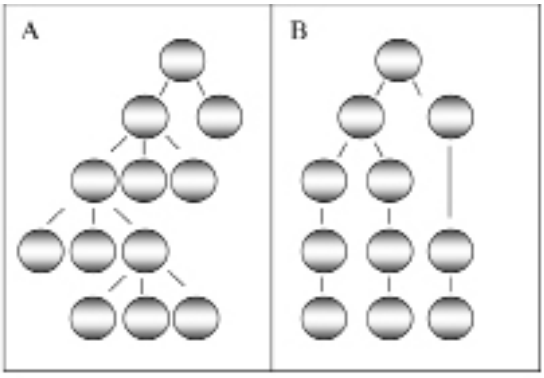

Figure 1: Intraclonal variation is not necessarily a reflection of ongoing SHM. Schematic representation of lymphoma cells where continuous AID expression induces ongoing SHM resulting in IgV_H variation between tumor subclones (A). However, the presence of ICV may also be a sign of SHM activity in the past. In B, AID expression during/shortly after malignant transformation has resulted in ICV. This ICV can still be detected, even when AID expression has stopped and therefore SHM is no longer ongoing. Adapted from R.J. Bende.

In chapter two, three and six we describe that there is no correlation between AID expression, total number of consensus mutations and intraclonal variation. These data were supported by nearly all investigating groups [22;23;25]. Whereas we did find ASHM in *C-MYC*, *PIM1* or *BCL6* in AID-expressing DLBCL (n=4 unpublished data), these genes were unmutated in those FL with proven SHM activity. Pasqualucci *et al.* describe that 74% of the ASHM$^+$ DLBCL express AID, although there was no direct correlation between AID protein amount and total number of mutations [30]. The lack of correlation between AID expression and (A) SHM in lymphomas, as also described by Lossos *et al,* represents the heterogeneity between different lymphomas [23]. In fact, based on the activity of the SHM machinery three lymphoma subgroups can be distinguished.

	Somatic hypermutation			
Lymphoma	**IgV $_H$**	**ICV**	**ASHM**	**AID**
MCL	30%	-	nd	-
BL	+	+	30%	+
HL	+	+	55-80%	+
FL	+	+	30%	25%
DLBCL	+	+	47 - 100%	32%
MBCL	+	-	74%	+
MZBCL	+	+	76%	-
B-CLL	50%	60%	0%	-
HCL	+	73%	nd	+
PEL	+	14%	66%	nd
MM	+	-	nd	-
LPL	+	-	nd	+

Table 1: Somatic hypermutation activity in B-NHL. ASHM concerns the percentage of lymphomas that shows mutations in one of the following genes: PAX5, TTF/Rho, PIM1 or C-MYC. The variability of ASHM in DLBCL is due to differences between different studies. The variability of ASHM in HL is due to differences between HL subtypes with 80% ASHM of lymphocyte predominant HL and 55% ASHM of classic HL. ASHM; aberrant somatic hypermutation, BL; Burkitt's lymphoma, B-CLL; chronic lymhocytic leukemia, DLBCL; diffuse large B cell lymphoma, FL; follicular lymphoma, HCL; hairy cell leukemia, HL; Hodgkin's lymphoma, LPL; lymphoplasmacytic lymphoma, MZBCL; marginal zone B cell lymphoma, MBCL; mediastinal large B-cell lymphoma, MCL; mantle cell lymphoma, MM; multiple myeloma, nd; not determined PEL; primary effusion lymphoma. References:[3;6;10;30;30;32-44]

The first subgroup has been mentioned already and consists of lymphomas with ongoing SHM activity like BL, and a subset of FL and DLBCL. These lymphomas express AID, show ICV that increases in time and acquire new IgV_H SHM in time. However, most of the FL and

DLBCL belong to the second subgroup. These lymphomas express mutated IgV_H genes with ICV, but do not express AID. Continuous activity of the SHM machinery may endanger the expression of a functional BCR, which may favor the outgrowth of AID negative subclones. Another interesting hypothesis is based on the fact that PAX5 is a key regulator for AID expression [31]. Since targeting of the region involved in initiation of translesion is described in one DLBCL [32] ASHM may thus limit its own activity. A third subset of lymphomas expresses AID but do not have ICV. The presence of IgV_H mutations shows that the mutation machinery has at least been active before. AID may have become inactive due to a variety of reasons like mutations, subcellular localization or missing/mutated co-factors. Or alternatively, similar to the RS B-CLL, as described in chapter three, the SHM machinery may target non-*Ig* genes while leaving the IgV_H genes intact. UNG and several members of the mismatch repair machinery have now been described to be targeted by low levels of SHM in normal B cells. Since the targeting of the SHM machinery may be determined by the action of error-prone and high-fidelity repair [16], gene expression levels and mutation analysis of these genes in lymphomas may provide more insight into their role in aberrant somatic hypermutation during lymphomagenesis.

AID in B-CLL

Approximately 50% of B-CLL express mutated IgV_H genes indicative for passage through a germinal centre reaction [45]. Microarray analysis showed that both the IgV_H-mutated and IgV_H-unmutated B-CLL have a gene expression profile comparable to that of memory B-cells[46]. In chapter two and three we describe that, in accordance with this phenotype, peripheral blood B-CLL cells and B-CLL lymph node samples do not express detectable levels of AID. Our data seem to contradict other reports that describe AID expression in mostly unmutated B-CLL [24;47-50]. However, these studies were performed using non-quantitative RT-PCR methods on B-CLL samples of unknown purity, whereas western blot analysis [22], quantitative PCR analysis [23] and limiting dilution assays [51] showed AID expression that was confined to at most a minority (0,01-0,2%) of the tumor clone. We describe in chapter three that *in vitro* stimulation can induce AID expression in B-CLL cells. Others have described that appropriate *in vitro* stimulation can induce IgV_H mutations in B-CLL cells, indicating that they have retained their capacity to undergo SHM [52]. IgV_H mutation analysis showed a low but significant intraclonal variation in 25% of the mutated B-CLL [49;52]. Nevertheless, since AID is not expressed in the (mutated) B-CLL subset, the observed ICV seems a reflection of a previous active SHM machinery. Furthermore, IgV_H mutation analysis of both mutated and unmutated B-CLL cells in time showed a decrease of ICV and no accumulation of mutations[53-55]. This suggests that while B-CLL cells have retained the capacity to undergo SHM, their IgV_H genes are not targeted by the SHM machinery.

AID in the progression of low grade lymphomas to high grade lymphomas

As mentioned above, AID is required for the occurrence of pristine-induced *IgV_H/C-MYC* translocations in BALB/cAn mice [17,56]. These translocations are not sufficient to cause lymphomas indicating that additional genetic hits are necessary. Interestingly, in the absence of AID, the number of translocation positive cells was greatly diminished after the pristine-induced occurrence of tumors. Apparently, AID promotes outgrowth of translocation positive cells [56]. This has led the authors to hypothesize that AID plays a role in tumor progression. However, although we agree that these experiments show that AID is necessary to complete malignant transformation of the translocation positive cells, we think this reflects tumor initiation rather than tumor progression. Unfortunately, to our knowledge this is the only paper that even mentions the role of AID in tumor progression. As of today, no appropriate mouse models are described to investigate the role of AID in lymphoma progression.

In vivo data that correlate AID expression with poor clinical outcome suggest, perhaps only indirectly, that AID is indeed instrumental in lymphoma progression. Activated B-cell like DLBCL that express higher levels of AID show a poorer overall survival than the germinal centre B-cell like DLBCL [23;44;57]. Also primary cutaneous large B-cell lymphomas (PCLBCL) with an intermediate prognosis express higher levels of AID than primary cutaneous follicle centre lymphomas (PCFCL) with an indolent behavior [44]. This observation is consistent with the fact that PCLBCL and PCFCL have gene expression profiles similar to those of germinal centre B-cell like DLBCL and activated B-cell like DLBCL respectively [58]. Also, the occurrence of ASHM seems to be a frequent feature of the more aggressive lymphomas. AIDS-associated-FL, -BL and -PEL with their aggressive behavior carry more ASHM than FL and BL of the immunocompetent hosts [30]. In addition, DLBCL of the central nervous system with their poor prognosis show a 2 to 5-fold increase of ASHM in comparison to the extracerebral DLBCL [32]. Altogether, it seems that ASHM is a frequent feature of the more aggressive lymphomas.

In chapter two, three and four we investigated whether the SHM machinery played a role in tumor progression of B-LBL, FL and B-CLL. In chapter two, we describe an increase in AID expression in 3 of the 7 investigated FL after histological transformation. These findings were in accordance with the findings of the group of Lossos *et al.* who showed an increase in AID expression in 3 of the 12 investigated FL and stable AID expression in 2 FL in time [23]. Histological transformation is associated with the acquisition of new mutations in the translocated *BCL2* gene [59] or the 5'coding region of *BCL6* [60]. Furthermore, ASHM was observed in 5 of 9 investigated DLBCL, whereas these mutations were not present in the preceding FL [61]. Unfortunately, AID expression was not measured in these lymphomas, but it seems that clonal selection results in outgrowth of subclones in which the SHM machinery had been active.

In B-CLL, we could not quantify AID expression in any of the mutated or unmutated cases. However, 4 of the 5 B-CLL that were about to undergo histological transformation to a DLBCL, did express measurable levels of AID. Furthermore, we show that 4 out of 5 investigated RS B-CLL showed ICV in the IgV_H and/or *BCL6* gene, which indicates activity of the SHM machinery. Reiniger *et al.* reported an increase in AID expression after Richter transformation and prolymphocytic transformation [43]. Several reports have described an increase in the frequency of ASHM upon Richter's transformation [43;61]. It has been described that Richter transformation is accompanied by an increase of chromosomal imbalances and microsatellite instability [62;63]. Schatz et al. showed that it is not the mistargeting of AID that underlies ASHM, but rather the breakdown of protective high fidelity repair. The role of AID in tumor progression may be twofold. Genetic instability may occur via ASHM of proto-oncogenes that result in chromosomal translocations, and/or AID may introduce mutations in members of the DNA repair pathways which may add to the genetic instability observed in high grade lymphomas.

The microenvironment in FL and B-CLL

For their proliferation, survival, (re-)circulation through lymphoid tissues and their antibody response, B-cells are dependent on appropriate stimuli from their microenvironment. Extensive evidence indicates that the microenvironment also plays a crucial role in the onset and growth of a variety of B-cell malignancies. As reviewed in chapter six, FL express a structurally intact BCR, even though the somatic hypermutation load is high and in some cases ongoing, which suggests that they may still bind antigen[28;64;65]. FL cells immediately undergo apoptosis when cultured in vitro. Furthermore, in vivo they still grow in a dense network of non-malignant T cells, macrophages, FDCs and other accessory stromal cells [66]. Furthermore, microenvironmental factors can have prognostic values. An increased number of macrophages has been associated with poor progression free survival [67]. An increased number of regulatory T cells (Treg) has been correlated with improved overall survival[68]. Interestingly, upon morphological transformation of the FL into a higher grade FL or even DLBCL, the microenvironment alters as well. Whereas FDCs in FL show a phenotype similar to normal GC FDCs, upon transformation the expression of CD21, CD22 and CD35 is lost and the FDCs express a more stromal phenotype[69]. Loss of differentiated FDCs was associated with a decreased number of intrafollicular $CD3^+$ T cells [69]. However, whether this is merely a reflection of the more autonomous growth pattern of the high grade lymphoma or whether this indeed is instrumental in tumor transformation is currently unclear.

In B-CLL increased numbers of $CD4^+$ and $CD8^+$ T cells have been reported. A subset of the $CD4^+$ T cells also contained perforin (PF), which could indicate cytolytic potential. It has been suggested that these T cells may play a role in an anti-tumor response. On the other

hand, $CD4^+$ PF^+ T cells also produce IL4, which has been described to protect B-CLL cells against apoptosis. In addition to IL4, activated $CD4^+$ T cells can secrete IL2, TNFα, GM-CSF and IL6, which also support the B-CLL cells. Interestingly, $CD4^+$ T cells from B-CLL patients with more progressive disease were more prone to produce cytokines than B-CLL patients with indolent disease [70]. Furthermore, part of the B-CLL express B-lymphocyte activating factor (BAFF) that can stimulate B-CLL cells in an autocrine fashion, and also T cell proliferation and survival [70]. In addition, CD40/CD40L interaction by B-CLL/$CD4^+$ T cell respectively, induces secretion of CCL22, which induces the migratory potential of CCR4-expressing $CD4^+$ T cells towards the B-CLL cells [71]. Furthermore, as described in chapter seven, the CD40/CD40L interaction induces an increase in survivin, BCL-xL and A1 and a decrease in the pro-apoptotic Noxa, which altogether accounts for an anti-apoptotic gene expression profile.

Multi-agent treatment of B-CLL can result in a profound peripheral lymphocyte depletion whereas the lymphocytes in the bone marrow and lymph nodes are less effectively targeted. Persistence of CLL cells in these lymphoid organs is associated with an increased risk of relapse. However, despite this well known fact, the majority of experiments concerning the stimulatory B-CLL/T cell interactions and B-CLL/DC interactions were performed on peripheral blood cells. In chapter seven significant gene expression differences between peripheral blood B-CLL cells and lymph node B-CLL cells are described, which shows that future research concerning the biology of B-CLL should focus on this highly specific microenvironment. In addition, little is known about microenvironmental alterations during Richter's transformation. As we describe in chapter three, structural BCR analysis suggests that B-CLL undergoing Richter's transformation may recognize recurrent antigenic epitopes. Furthermore, despite low but active somatic hypermutation machinery during Richter's transformation the structural integrity of the BCR remains intact, which may suggests that antigen is still recognized after transformation. However, we also show that the DLBCL cells become less responsive to CD40 stimuli. Further analysis of the microenvironmental alterations during Richter's transformation would provide more insight into the influence of the microenvironment in transformation and may, in time, provide for additional therapeutic targets.

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Summary
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Summary

The germinal centre is a specialized microenvironment in which B cells, T cells and FDCs cooperate to constitute an effective humoral immune response. The outcome of this process is the production of high affinity antibodies by the B cells. Somatic hypermutation involves the introduction of mostly point mutations in the variable region of the Ig genes thus altering the affinity of the Ig for the invading antigen. However, SHM also endangers the genomic integrity of the rapidly proliferating germinal centre B cell by the introduction of mutations in non-Ig genes including tumor suppression genes and proto-oncogenes. Aberrant SHM (ASHM) may also lead to the formation of double stranded DNA breaks and (proto-) oncogene chromosomal translocations. SHM and specifically ASHM is assumed to play a role in the initiation of a variety of B-NHL. In this thesis we investigated whether or not this potentially harmful process is still active in B-NHL and as such is instrumental in the progression of low grade to high grade lymphomas.

In **chapter one** a general introduction is given on the humoral immune response, B-cells, the germinal centre and in particular somatic hypermutation and the enzyme that plays a key role in this process, activation induced cytidine deaminase. Furthermore, we give a short introduction about the genetic alterations that characterize certain subsets of B-NHL and describe how SHM may play a role in B-cell lymphomagenesis.

In **chapter two**, we quantitatively measured the expression of AID and polymerase iota in a comprehensive panel of B-NHL. AID was found to be only expressed in B-NHL with a germinal centre phenotype. AID expression did not correlate with IgV_H intraclonal variation, indicating that the general believe that ICV is a reflection of ongoing SHM is not always true and ICV in fact may be a remnant of previous SHM activity in the GC. In this chapter we also describe seven FL with histological progression to a DLBCL. Three out of seven FL showed an increase in AID expression level after transformation, which suggests that ongoing SHM activity may have played a role in tumor progression.

In **chapter three** these topics were further addressed in a specific subgroup of low grade lymphomas, i.e. B-CLL. We show that in accordance with the data presented in chapter one, B-CLL do not express AID. However, B-CLL that undergo transformation to a diffuse large B cell lymphoma expressed considerably higher levels of AID. Furthermore, in three out of five DLBCL, AID expression was accompanied by ICV of IgV_H or *BCL6* and/or an increase in mutations in IgV_H or *BCL6.* We also show in this chapter that these B-CLL express remarkably homologous IgV_H-CDR3 regions, suggesting that these lymphomas may recognize recurrent antigenic epitopes.

In **chapter four** we describe three cases of FL that undergo progression to a precursor B-lymphoblastic lymphoma. After transformation the B-LBL did not only posses an immature

B cell immunophenotype, with Tdt, VpreB and λ5 expression, but also evidence of was obtained for ongoing immunoglobulin light chain rearrangement. *Ig* gene analysis further showed extensively mutated IgV_H genes, which was not ongoing during transformation. The clonal relation between these two tumors confirms the notion that in all three cases indeed differentiation to an earlier cell program had taken place. Finally, we show that one B-LBL gained a *BCL6* translocation, whereas all three B-LBL gained a *C-MYC* translocation after transformation.

In **chapter five** we describe four FL of the gastro-intestinal tract. These rare lymphomas expressed extensively mutated IgV_H genes and three of the GI-FL expressed IgA. Mutation analysis showed that in these GI-FL counterselection for potential harmful replacement mutations in FR had taken place. Furthermore, all lymphomas expressed α4β7, a well defined mucosal homing receptor, which may explain their low metastazing potential. Altogether, it seems that these GI-FL may originate from local, antigen experienced GC B cells and that their BCR may still have binding capacity for antigens originating from the gut lumen.

Chapter six summarizes existing data about the somatic hypermutation process in FL initiation and progression and sheds some light on the role of the microenvironment, particularly the B-cell receptor ligands, in lymphomagenesis in mice and men.

In **chapter seven** we investigated the role of the microenvironment in the pathogenesis of B-CLL. We compared the gene expression pattern of a large panel of apoptosis regulators of peripheral blood B-CLL cells and compared them with lymph node B-CLL cells. We found that, although the overall expression pattern between peripheral blood and lymph node B-CLL was remarkably similar, some genes were expressed differently. B-CLL isolated from lymph node samples expressed increased levels of anti-apoptotic BCL-xL and Survivin, and a decreased level of the pro-apoptotic BH3-only protein Noxa. We show that these gene expression differences are most probably due to sustained CD40L stimulation in the lymph node microenvironment, since CD40L-stimulation of peripheral blood B-CLL cells resulted in a similar gene expression pattern. Furthermore, Bortezomib-induced Noxa expression resulted in increased apoptosis, whereas decreased Noxa expression by means of RNAi, reduced bortezomib-induced apoptosis. These data indicate that suppression of Noxa in the lymph node microenvironment contributes to the persistence of B-CLL at these sites.

Finally, **chapter eight** summarizes and discusses the results presented in this thesis. We compare our quantitative AID expression level data with those presented by other groups. We describe that in contrast to the general believe, the SHM machinery is active in only a subset of FL and that ICV is not necessarily a reflection of ongoing SHM. Furthermore, we compare our data concerning ongoing SHM in B-NHL with those described in literature and conclude that although still only limited data exist, SHM may indeed play a role in lymphoma progression.

Samenvatting

Het kiemcentrum is een gespecialiseerde micro-omgeving waarin B cellen, T cellen en folliculair dendritische cellen samenwerken om een effectieve humorale immuunrespons te bewerkstelligen. De uitkomst van dit proces is de productie van antistoffen door de B cel met een hoge affiniteit. Somatische hypermutatie introduceert voornamelijk punt mutaties in de variabele regio van de immunoglobuline genen om zo de affiniteit van de Ig voor het binnendringende antigen te veranderen. SHM kan echter ook de genomische integriteit van de snel delende kiemcentrum B cel in gevaar brengen doordat er mutaties in niet-Ig genen (waaronder tumor suppressor- en oncogenen) worden geïntroduceerd. Deze aberrante SHM (ASHM) kan ook leiden tot de vormingen van dubbelstrengs DNA breuken en chromosomale translocaties in (proto)oncogenen. Het lijkt erop dat SHM en met name ASHM een rol speelt in het ontstaan van een aantal B-NHL. In dit proefschrift hebben we onderzocht of dit potentieel gevaarlijke proces nog aanstaat in B-NHL en zo een rol speelt in de progressie van laaggradige naar hooggradige lymfomen.

In **hoofdstuk een** wordt er een algemene inleiding gegeven over de humorale immuun respons, B cellen, het kiemcentrum en met name het enzym dat een hoofdrol speelt in SHM, activation induced cytidine deaminase. Verder geven we ook een korte introductie in de genetische afwijkingen die kenmerkend zijn voor bepaalde B-NHL en beschrijven we hoe SHM een rol zou kunnen spelen in het ontstaan van B-NHL.

In **hoofdstuk twee** hebben we de expressie van AID en polymerase iota kwantitatief gemeten in een uitgebreid panel van B-NHL. AID bleek alleen tot expressie te komen in lymfomen met een kiemcentrum fenotype. AID correleerde niet met de IgV_H intraklonale variatie, wat erop wijst dat het algemeen aanvaarde principe dat ICV een uiting is van doorgaande SHM niet correct is en dat ICV ook een restant kan zijn van vroegere SHM activiteit in het kiemcentrum. In dit hoofdstuk beschrijven we zeven FL met histologische progressie naar een DLBCL. Drie van de zeven FL brachten meer AID tot expressie na transformatie, wat erop wijst dat SHM een rol gespeeld zou kunnen hebben in de tumor progressie.

In **hoofdstuk drie** worden deze onderwerpen verder besproken in de specifieke subgroep van laaggradige lymfomen nl. B-CLL. We laten zien dat, in overeenstemming met de data uit hoofdstuk een, B-CLL geen AID tot expressie brengen. Echter, B-CLL die progressie doormaken naar een DLBCL brengen duidelijk meer AID tot expressie. Bovendien was de AID expressie in drie van de vijf DLBCL gecorreleerd aan ICV in IgV_H of *BCL6* of een toename van mutaties in deze genen. We laten in dit hoofdstuk ook zien dat deze B-CLL opvallend homologe IgV_H –CDR3 regio's tot expressie brengen, wat suggereert dat deze lymfomen specifieke, steeds terugkerende antigenen herkennen.

In **hoofdstuk vier** beschrijven we drie FL die progressie doormaken naar een precursor B

cel lymfoom . Na transformatie brengen deze lymfomen niet alleen een immatuur B cel immunofenotype tot expressie met TdT, VpreB en lambda 5, maar ze gaan ook hun lichte genen opnieuw herschikken. Ig gen analyse laat zien dat hun IgV_H genen gemuteerd zijn, maar dat deze SHM niet doorgaat in de lymfomen. Het feit dat de lymfomen voor en na transformatie dezelfde klonale IgV_H genen tot expressie brengen wijst erop dat in alle drie de gevallen differentiatie naar een vroeg B cel programma is opgetreden. Tot slot laten we zien dat één lymfoom een BCL6 translocatie en alle drie de lymfomen een C-MYC translocatie hebben gekregen na transformatie.

In **hoofdstuk vijf** beschrijven we 4 gastro-intestinale FL. Deze zeldzame lymfomen brengen gemuteerde IgV_H genen tot expressie en drie GI-FL produceerden IgA. Mutatie analyse laat zien dat er in deze lymfomen selectie heeft plaatsgevonden tegen potentieel schadelijke mutaties in de framework regio van de Ig. Verder brachten alle lymfomen α4β7 tot expressie, een mucosale homing receptor, wat zou kunnen verklaren waarom deze lymfomen zich zo weinig door het lichaam verspreiden. Samengevat lijkt het erop dat deze lymfomen van lokale kiemcentrum B cellen afstammen die met antigeen in contact zijn geweest en dat hun Ig nog steeds antigenen herkennen in het maag/darm kanaal.

Hoofdstuk zes is een samenvatting van bestaande data over SHM in het ontstaan en de progressie van FL. Bovendien behandelt het de rol van de micro-omgeving en met name B cel receptor liganden in de lymfomagenese in mensen en muizen.

In **hoofdstuk zeven** hebben we de rol van de microomgeving in de pathogenese van B-CLL onderzocht. We hebben gen expressie patronen van een groot aantal apoptose regulatoren van B-CLL cellen in het bloed vergeleken met die in de lymfklieren. Het blijkt dat deze genexpressie patronen opvallend overeenkomstig waren. B-CLL cellen uit de lymfklieren brachten echter meer van het anti-apoptotische BCL-xL en survivin tot expressie en minder van het pro-apoptotische Noxa. We laten zien dat deze verschillen worden veroorzaakt door CD40 stimulatie in de lymfklier omgeving, aangezien CD40 gestimuleerde bloed B-CLL cellen eenzelfde gen expressie patroon kregen. Verder leidde Bortezomib geïnduceerde opregulatie van Noxa tot togenomen apoptose en RNAi geïnduceerde verlaging van Noxa tot verminderde Bortezomib geïnduceerde apoptose. Deze data wijzen erop dat Noxa in de lymfklier omgeving bijdraagt aan het overleven van de B-CLL cellen hier.

Tot slot worden de data uit dit proefschrift in **hoofdstuk acht** samengevat en bediscussieerd. We vergelijken onze AID expressie data met die van andere onderzoekers. We beschrijven dat ICV in tegenstelling tot wat altijd werd gedacht geen teken is van doorgaande SHM. Bovendien vergelijken we onze SHM data in B-NHL met bestaande literatuur en concluderen dat hoewel er nog maar weinig over bekend is, SHM inderdaad een rol speelt in de progressie van B cel lymfomen.

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Curriculum vitae

Laura Angelique Smit is geboren op 19 februari 1977 te Amsterdam. In 1995 haalde zij haar VWO diploma aan het Waterlant College te Amsterdam. Hierna is zij Medische Biologie gaan studeren aan de Universiteit van Amsterdam. In 1997 werd ze ingeloot voor de studie geneeskunde en heeft ze beide studies aan de UvA gevolgd. Tijdens haar studies heeft ze twee wetenschappelijke stages gevolgd bij de afdeling Pathologie op het AMC te Amsterdam onder begeleiding van prof. dr. Carel van Noesel. De eerste stage was in samenwerking met de gynecologie onder leiding van prof. M. Burger en ging over het indentificeren van HPV subtypen bij cervixcarcinomen. Het was echter de tweede stage die haar interesse voor de immunologie aanwakkerde. Mede begeleid door Richard Bende heeft zij onderzocht welke genen Folliculair Dendritische Cellen tot expressie brachten. Dit beviel haar zo goed dat zij, nadat ze in april 2001 haar doctoraal geneeskunde had behaald en in oktober 2001 haar doctoraal Medische Biologie, begon als AIO bij de afdeling Pathologie op het AMC. Onder leiding van prof. dr. Carel van Noesel en begeleid door Richard Bende heeft ze 4,5 jaar onderzoek gedaan naar de rol van basale immunologische processen zoals somatische hypermutatie in de progressie van laaggradige B-cell non Hodgkin's lymfomen. De resultaten hiervan zijn in dit proefschrift beschreven. Van februari 2006 tot en met februari 2008 heeft zij haar co-schappen gelopen en in maart 2008 haar artsexamen behaald. Momenteel is zij bezig met haar opleiding tot Patholoog in het AMC te Amsterdam.

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