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**REVIEW**

# Molecular pathways in follicular lymphoma

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**Follicular lymphoma (FL) is one of the most common B-cell non-Hodgkin's lymphomas. The initiating genetic event found in ~90% of FL is the t(14;18), causing constitutive expression of the antiapoptotic 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.**
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## 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 an FL variant, although this entity generally lacks the characteristic t(14;18)(q32;q21).[1,3] Surprisingly, primary gastro-intestinal 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 naive 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 costimulatory 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 owing 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 FLs most often display an exclusive centroblastic morphology, may be BCL-2$^-$ and are classified by the WHO as grade 3B.[1]

## Somatic Ig gene alterations and lymphomagenesis

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. SHM alters the affinity of the $IgV_H$ and $IgV_L$ chains for the antigen (Ag). Mutated subclones with the highest affinity for Ag

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will retain survival signals elicited by Ag and finally predominate the GC reaction. Subsequently, the Ag-selected B cells may undergo Ig 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 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 owing to erroneous CSR have been found, for example, *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 owing 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, that is, 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,[42,57] whereas aberrant SHM was not found in *PIM-1*, *C-MYC* and *PAX-5* and found rarely in *RhoH/TTF* (see Pasqualucci *et al*.[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.[58,59] 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.[60,61]

Several investigators believe that, given their strong resemblance to normal GCs, FLs have retained essential functional properties of their non-neoplastic counterparts as well, in particular the capacity to actively hypermutate their *IgV* genes. This idea was supported by the observation of so-called

**Table 1** Chromosomal translocations of mature B-NHL

| *Lymphoma* | *Translocation* | *Involved genes* | *Proposed mechanism* | *% of cases (references)* |
|---|---|---|---|---|
| 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%[44] |
| | t(3;14)(q27;q32) | BCL-6/IgH | CSR | ∼5%[17,18 a] |
| MZBCL | t(11;18)(q21;q21) | API-2/MALT | ? | [45–47 b] |
| | t(1;14)(p22;q32) | BCL-10/IgH | CSR | ∼5%[45] |
| | t(14;18)(q32;q21) | IgH/MALT | CSR | [45 c] |
| | t(3;14)(p14.1;q32) | FOX-P1/IgH | CSR | [48–52 d] |
| DLBCL | t(14;18)(q32;q21) | IgH/BCL-2 | V(D)J | 15–30%[53 e] |
| | t(3;14)(p14.1;q32) | FOX-P1/IgH | CSR | [48–52 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%[54] |
| | t(8;22)(q24;q11) | C-MYC/Igλ | SHM | ∼5%[54] |
| BL | t(8;14)(q24;q32) | C-MYC/IgH | CSR/SHM | ↓ |
| | t(8;22)(q24;q11) | C-MYC/Igλ | SHM | BL together 100%[55,56] |
| | 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: BL, Burkitt's lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; MZBCL, marginal zone B-cell lymphoma.
[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.[45–47]
[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.[45]
[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.[48–52]
[e]t(14;18) is merely found in the GC B type of DLBCL.[54]

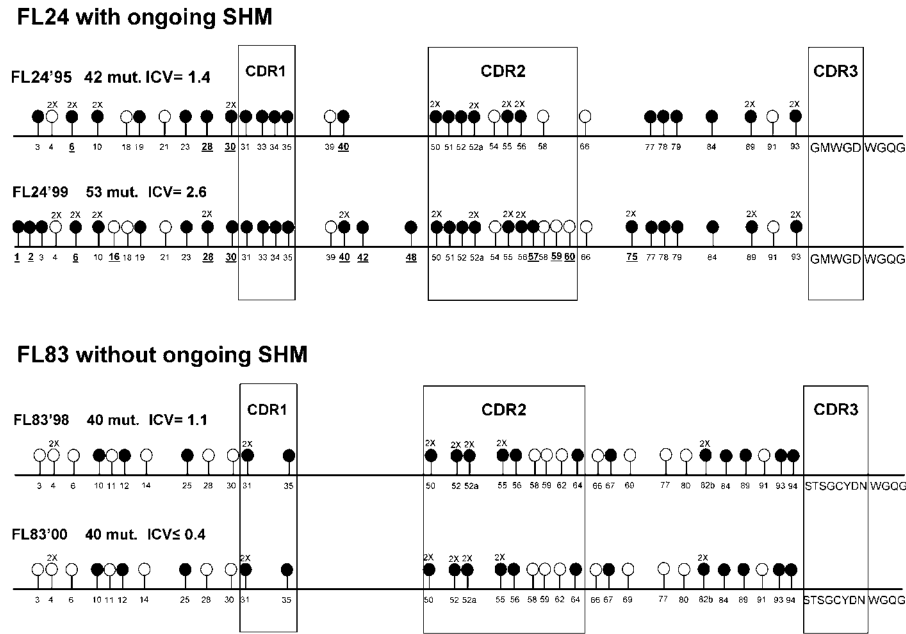


**Figure 1** Serial biopsies of two FLs, with and without ongoing Ig$V_H$ SHM. Schematic representation of Ig$V_H$ consensus sequences including somatic mutations, obtained from serial biopsies of FL24 and FL83. The lollipop-shaped symbols indicate nucleotide differences as compared with the V3–23 germline *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 Ig$V_H$ mutations as well as the ICV increased over time, compatible with ongoing SHM. In contrast, in FL83 the total number of Ig$V_H$ 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.[65] At least seven individual Ig$V_H$ clones were sequenced of the two time points of both FLs. The ICV is calculated as the mean number of additional Ig$V_H$ mutations of the individual molecular Ig$V_H$ clones, as compared to the consensus Ig$V_H$ sequence of a given FL.

intraclonal IgV nucleotide variation (ICV) among tumor subclones. Significant ICV is indeed found in the vast majority of FLs.[60] However, based on analyses of sequential biopsies of a total of nine FLs, spanning significant periods, we questioned whether ICV is indeed the result of ongoing SHM.[60,62–64] In only two out of these nine FLs, we observed evidence compatible with ongoing SHM, that is, ICV at both time points, accumulation of the total number of Ig$V_H$ mutations over time and sustained AID expression (Figure 1).[60,65] One FL showed no ICV at either time points, whereas six FLs showed a decrease in ICV over time, without clear accumulation of the total number of Ig$V_H$ mutations. (Figure 1).[60,62–64] In three FLs, evidence was obtained for the selective outgrowth of minor subclones, that had been present already at the earliest time points.[63,66,67] 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.[65,68,69] Thus, as AID is essential for SHM, ICV alone cannot be taken as a measure for ongoing SHM.

## The role of the B-cell antigen receptor complex in FL development

During all phases of normal development, B cells are 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 owing 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 Ig$V_H$ and Ig$V_L$ 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 Ig$V_H$-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 owing 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 contain 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.[60,76,77] For comparison, in ~40% of DLBCLs such counterselection for R mutations in FRs is not found. Accordingly, about half of DLBCLs lack BCR protein expression, indicating that they are less dependent on BCR signals than FLs are.[78]

As the BCRs are structurally intact in FLs, they may still bind Ag which is most likely present at the surface of non-neoplastic FDC in the follicles. However, immunohistochemical staining using four 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*.[80] eight out of 31 FL-derived Igs reacted *in vitro* with nuclear-Ags, IgG, actin and tubulin. 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.[59,60,79,81] Moreover, the mean IgV$_H$-CDR3 amino-acid sequence length of FL is comparable to that of normal naive B cells.[79,82] As 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 with 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 their 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 polymerase chain reaction, evolve in the gut mucosa, express the mucosal homing integrin $\alpha 4\beta 7$, 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, for example, 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 in 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 is being selected for during FL development. Supra-physiological *N*-glycosylation may enhance Ag-independent BCR signaling (tonic signaling) or, alternatively enhance specific or nonspecific 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, DCs, macrophages, 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 four 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 interleukin-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 tumor necrosis factor (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, LT$\alpha$ and LT$\beta$.[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, that is, 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%) absence of the established FDC antigens CD23, CD21, CD35 and with variable expression of LNGFR and CNA.42.[102] The FLs 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.[67]

## Secondary gene alterations in FL

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, as only a few secondary DNA alterations are known to occur in a substantial fraction of FLs, scenarios for stepwise transformation are still ill-defined. Höglund *et al*.[103] identified, by classical cytogenetics on 336 t(14;18)$^+$ FLs, 5 recurrent chromosomal alterations, each occurring in at least

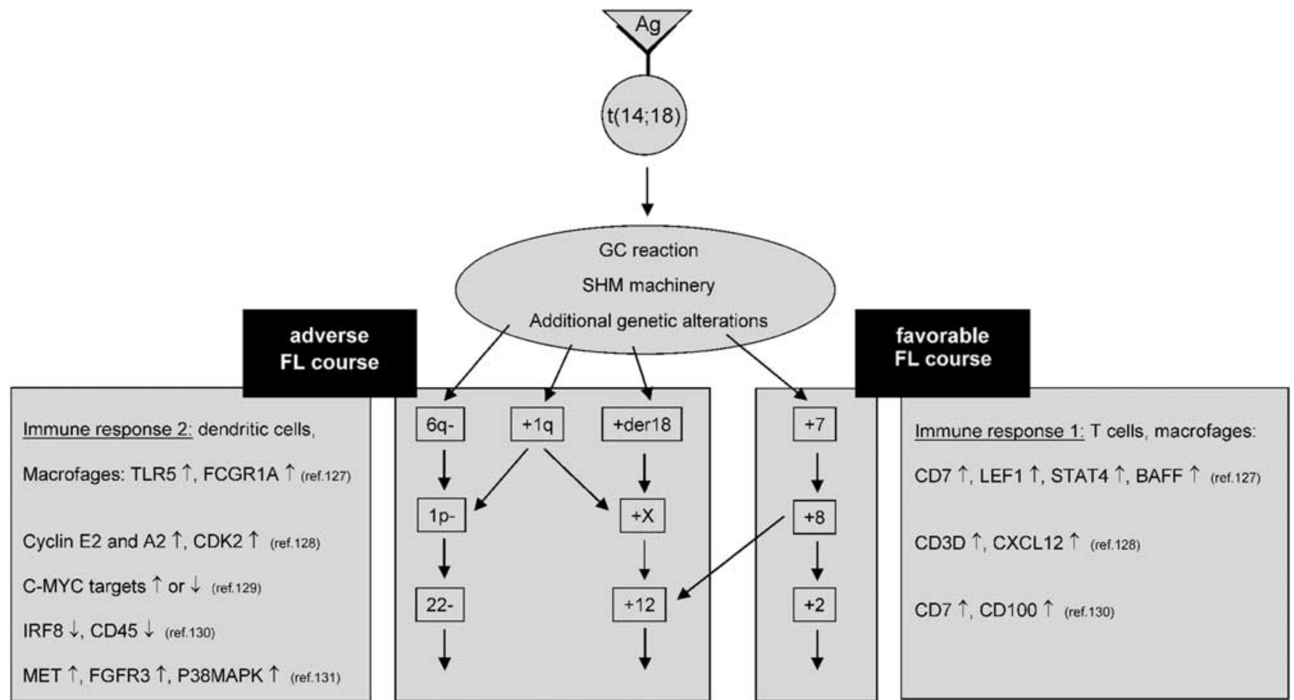


**Figure 2** The genetic pathways of FL in relation to the clinical course.

20% of the FL: −1p32–36 (20%), −6q11–27 (30%), +7 (28%), +12 (23%) and +X (24%). The mean number of imbalances was ∼6 per FL with most FLs having two 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, four 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 a univariate analysis of 165 FL patients of which complete clinical information was available, six 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]

The alterations affecting chromosome 1, that is, −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 (Fc$\gamma$R-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]

Owing 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 FLs 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 six 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* (TGF$\beta$ 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* (Fc$\gamma$R-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 FLs (histological grades 1–2) with those of corresponding biopsies of grade 3 FLs or FLs that had transformed into lymphomas 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* (CD3$\delta$), 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 threefold 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, whereas in four cases a decreased expression of *C-MYC* and its target genes was observed. De Vos *et al.*[130] studied four FLs with documented progression. Interestingly, of their top list of 36 upregulated and 66 downregulated genes upon transformation, seven 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 downregulation 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 $\beta$ receptor) and *PDGFRB* (platelet-derived growth factor receptor $\beta$). 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 four C-MYC targets were upregulated, that is, *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* (Figure 2). These good prognosis groups may coincide with FLs 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 DCs. 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 five 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 on 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 (E$\mu$), primarily developed follicular hyperplasia but not lymphoma.[10,11] After 1 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 owing 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-hematopoiteic 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 E$\mu$-*bcl-2/myc* transgenic mice generated lymphomas much faster than both single E$\mu$-*bcl-2* and single E$\mu$-*myc* transgenic mice. The lymphomas in double E$\mu$-*bcl-2/myc* transgenic mice consist most often of mIgM$^{-}$ lymphoblastic cells in which c-myc initiates robust proliferation, most likely the proapoptotic properties of c-myc are inhibited by bcl-2.[136] In the lymphomas of single E$\mu$-*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 E$\mu$-*myc* model is the counteraction of the proapoptotic capacities of c-myc. This can be achieved either by inhibition of the Arf-Mdm2-p53 proapoptotic tumor suppressor pathway or by overexpression of the antiapoptotic bcl-2 protein. Forced deletion of Arf or p53, as expected, accelerated lymphomagenesis in the E$\mu$-*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] E$\mu$-*myc* mice that are deficient for the proapoptotic 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 E$\mu$-*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 antiapoptosis 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 E$\mu$-*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]

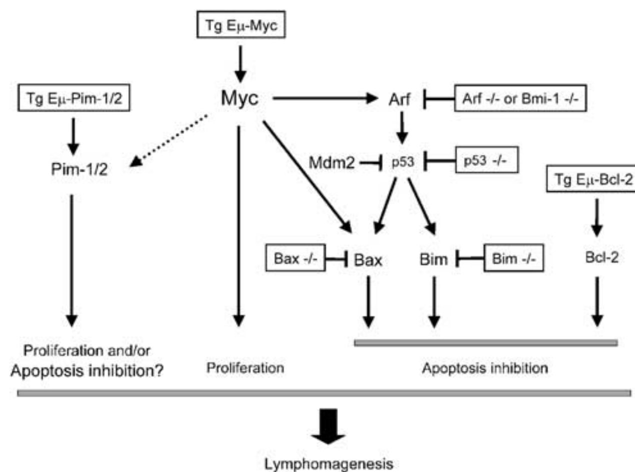


**Figure 3** Inhibition of c-Myc-induced apoptosis pathways accelerates lymphomagenesis in transgenic E$\mu$-myc mice. Additional molecular alterations introduced in E$\mu$-Myc mice are indicated in boxes; (Tg) transgenic or (–/–) knockout.

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 E$\mu$-*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 $V_H(D)J_H$-C$\mu$-C$\delta$ locus in a 'head to tail' orientation or from the Ig$\lambda$ 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 E$\mu$, 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, that is, ~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 E$\mu$-*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 I$\mu$ 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 DLBCLs contained clonal complex, nonrandom 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]

## 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 as 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

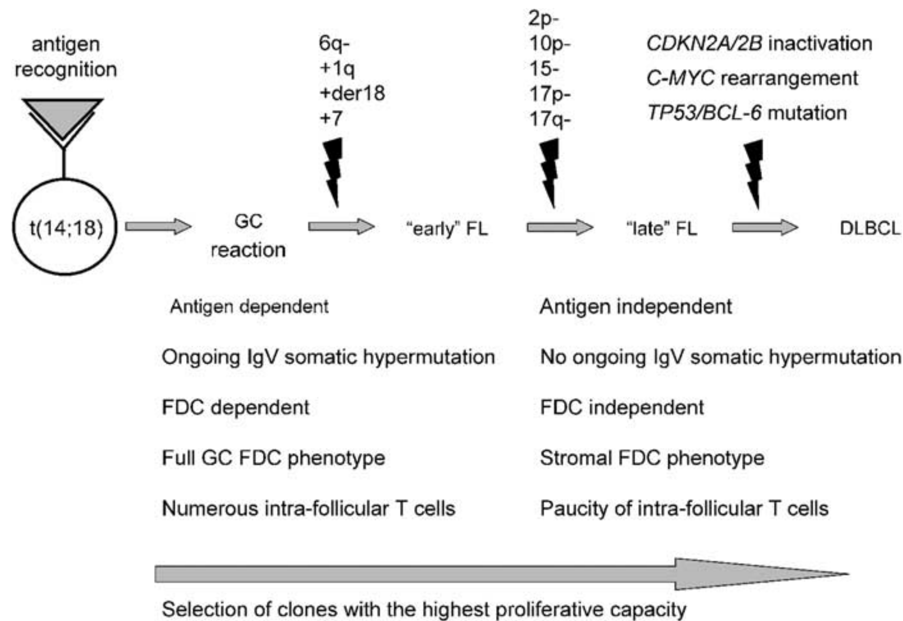


**Figure 4** The pathogenesis of FL.

hypermutating its *IgV* genes. FLs most likely arise randomly out of the pool of mature circulating t(14;18)$^+$ B cells, do not possess 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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