TUMORIGENESIS AND NEOPLASTIC PROGRESSION

B-Lymphoblastic Lymphomas Evolving from Follicular Lymphomas Co-Express Surrogate Light Chains and Mutated Gamma Heavy Chains

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Follicular lymphoma (FL) is an indolent B-cell non-Hodgkin lymphoma able to transform into germinal center-type diffuse large B-cell lymphoma. We describe four extraordinary cases of FL, which progressed to TdT⁺CD20[−] precursor B-lymphoblastic lymphoma (B-LBL). Fluorescence *in situ* hybridization analysis showed that all four B-LBLs had acquired a *MYC* translocation on transformation. Comparative genomic hybridization analysis of one case demonstrated that in addition to 26 numerical aberrations that were shared between the FL and B-LBL, deletion of *CDKN2A/B* and 17q11, 14q32 amplification, and copy-neutral loss of heterozygosity of 9p were gained in the B-LBL cells. Whole-exome sequencing revealed mutations in *FMN2*, *NEB*, and *SYNE1* and a nonsense mutation in *KMT2D*, all shared by the FL and B-LBL, and *TNFRSF14*, *SMARCA2*, *CCND3* mutations uniquely present in the B-LBL. Remarkably, all four FL–B-LBL pairs expressed IgG. In two B-LBLs, evidence was obtained for ongoing rearrangement of *IG* light chain variable genes and expression of the surrogate light chain. IGHV mutation analysis showed that all FL–B-LBL pairs harbored identical or near-identical somatic mutations. From the somatic gene alterations found in the *IG* and non-*IG* genes, we conclude that the FLs and B-LBLs did not develop in parallel from early t(14;18)-positive *IG*-unmutated precursors, but that the B-LBLs developed from preexistent FL subclones that accumulated additional genetic damage. (*Am J Pathol* 2016, 186: 3273–3284; <http://dx.doi.org/10.1016/j.ajpath.2016.07.027>)

Follicular lymphomas (FLs) are the prototypic germinal center (GC)-derived B-cell non-Hodgkin's lymphomas¹ and cytologically and histologically resemble normal GCs. In accordance, FLs share a gene expression profile with normal centroblasts and centrocytes² and express somatically hypermutated B-cell antigen receptors, which in approximately 25% of cases are of the non-IgM isotype.^{3,4}

FLs have an indolent clinical behavior with a reported median survival of 8 to 10 years after diagnosis.¹ However, 20% to 60% of FLs transform into higher-grade malignancies, an event associated with an aggressive clinical course, poor therapy responses, and short

survival.⁵ Usually, FLs transform into other GC-type lymphomas, most often diffuse large B-cell lymphomas (DLBCLs) or, less commonly, Burkitt-like lymphomas [referred to in the latest World Health Organization classification as B-cell lymphoma unclassifiable with features intermediate between DLBCL and Burkitt lymphoma (BL)].^{5,6} Infrequently, these prototypic GC-like malignancies transform into precursor B-lymphoblastic

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lymphomas (B-LBL).^{7–15} Studies on sporadic cases described so far revealed that such B-LBLs carried *BCL2* translocations, indicative for a clonal relationship with the preceding FLs. Karyotype analyses showed a variety of chromosomal alterations gained in the B-LBLs, among which *MYC* locus translocations were most frequently observed [in 18 of 19 (95%) of the described cases].^{7–15} Lymphomas with two recurrent translocations, one of which being *MYC*, are referred to as double-hit-B-cell lymphomas and have a dismal prognosis.⁶

Here, we analyzed four FLs that transformed into B-LBLs. We demonstrate that these B-LBLs phenotypically and functionally display key features of early B cells and that, in addition to *MYC* translocations, also *BCL6* gene translocation may accompany this infrequent mode of FL progression. Genome-wide copy number variation analysis and whole-exome sequencing (WES) of one of the transformed FLs demonstrated that a limited number of genomic alterations were gained in the B-LBLs, including deletions of the tumor suppressor genes *CDKN2A/B* and *NF-1* and mutations in *TNFRSF14*, *SMARCA2*, and *CCND3*.

Materials and Methods

Patient Material

The clinicopathologic characteristics of the four patients with a FL that transformed into a B-LBL are shown in Table 1. 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.

Table 1 Four Patients with a FL that Has Transformed into a B-LBL

Patient	Sex	Age, years	Diagnosis	Years from FL diagnosis	Location
TF1a	F	57	FL	0	Lymph node
TF1b			B-LBL	2	Epicardium
TF2a	F	51	FL	0	Lymph node
TF2b			B-LBL	1	Mouth
TF2c			B-LBL	2	Mouth
TF3a	F	24	FL	0	Lymph node
TF3b			FL	2	Lymph node
			B-LBL	2	Lymph node
TF4a	M	54	FL	0	Lymph node
TF4b			DLBCL	1	Abdominal wall
TF4c			B-LBL	1	Retroperitoneum

In TF1b, TF2b, and TF4b the preexisting 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.
Patients TF1, TF2, TF3, and TF4 all died within 2 years after FL diagnosis.
F, female; M, male; B-LBL, precursor B-lymphoblastic lymphoma; FL, follicular lymphoma.

Clinicopathologic History of the Four Patients

TF 1
A FL grade I was diagnosed, which was treated with chlorambucil. After 1 year a B-LBL was diagnosed, and the patient was treated with high-dose chemotherapy consisting of iphosphamide, etoposide, methotrexate, and two consolidation cycles with cytarabine, etoposide, and methotrexate. Complete remission was reached. A few weeks later progression of the disease occurred, and no further treatment was undertaken. The patient died subsequently.

TF 2
A FL grade I was diagnosed, which was not treated. Two years later a B-LBL was diagnosed, which was treated with daunorubicin, vincristine, prednisone, asparaginase, and methotrexate. Three months later the B-LBL reoccurred. The patient was treated with dexamethasone, but died a few weeks later.

TF 3
A FL grade I was diagnosed. Two years later, laboratory evaluation showed a leukocyte count of $10.7 \times 10^9/L$ with 38% atypical lymphocytes. Immunophenotyping demonstrated two lymphocytic populations, that is, one population of mature monoclonal B cells and a distinct population of lymphoid blasts. Combined chemotherapy with cytarabine, etoposide, and methotrexate was started. Complete remission was established after two additional courses of vincristine, dexamethasone, and adriamycin. Consolidation therapy consisted of cytarabine and asparaginase. Shortly thereafter the disease relapsed. Local radiotherapy was given. The patient died a few weeks later.

TF 4
A FL was diagnosed, which was treated with eight cycles of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone. A year later, positron emission tomography–computed tomography scan showed lesions in the abdominal wall and in the retroperitoneum, which pathologically turned out to be a DLBCL and a B-LBL, respectively. Rituximab combined with dexamethasone, high-dose cytarabine and cisplatin treatment was started, but eventually the patient died several months later.

Immunohistochemistry

Immunohistochemical stainings were performed on acetone-fixed cryostat sections and formalin-fixed paraffin-embedded tissue using Powervision⁺ detection system (Immunovision Technologies, Daly City, CA). Monoclonal antibodies used were CD20 (B-Ly1), CD21-L (DRC-1), BCL2 (124), BCL6 (PG-B6P), CD79a (HM57), CD19 (HD37), Igλ (A193), Igκ (A191) (Dako, Glostrup, Denmark); CD10 (CALLA) and PAX5 (24) [Becton and

Dickinson (BD), Erembodegem-Aalst, Belgium]; and CD3 (SP7; Labvision, Neomarkers, Fremont, CA). Terminal deoxynucleotidyl transferase (TdT) was stained with a polyclonal serum (Klinipath, Duiven, the Netherlands).

RNA Isolation, cDNA Synthesis

RNA was isolated using TRIzol reagent (Invitrogen, Breda, the Netherlands) and cDNA was synthesized.³ Immunoglobulin heavy chain variable region (IGHV) transcripts were amplified by PCR.^{16,17} In some experiments, IGHV-PCR products were cloned in pTOPO-TA-vectors and sequenced. IGHV genes were identified using V-Quest version 3.3 (http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanIg).¹⁸ VpreB and Lambda5 were amplified with the respective primer pairs: 5'-CTCATGCACTTTGTCTACTG-3'/5'-CTGGCTCTTGTCTGATTGTGAG-3' and 5'-CTCCTGTCCTGCTCATGCTG-3'/5'-GTACACACCGATGTCATGGTCG-3'. The *BCL2* PCR was performed on DNA, as described.¹⁹

FISH

Chromosomal aberrations were investigated in 4- μ m thick formalin-fixed paraffin-embedded tissue sections by fluorescence *in situ* hybridization (FISH). A segregation-detection assay was used to detect breaks in *BCL2* (probe Y5407), *BCL6* (probe Y5408), and *MYC* (probe Y5410) (Dako).

Cell Sorting

Lymph node suspensions of TF3b were stained with CD19-allophycocyanin, CD10-peridinin-chlorophyll protein complex-cyanine 5.5, and CD20-fluorescein isothiocyanate monoclonal antibodies (BD). Cells were sorted in small CD19⁺CD10⁺CD20^{lo} cells, small CD19⁺CD10⁺CD20^{hi} cells, small CD19⁺CD10⁻CD20^{hi} cells, and large CD19⁺CD10⁺CD20^{lo} cells by using a FACS Aria (BD).

Comparative Genomic Hybridization Array

Genomic DNA was isolated using DNeasy kit (Qiagen, Hilden, Germany) and analyzed for copy number variation using HumanCytoSNP-12 BeadChips (Illumina, San Diego, CA). Processing of DNA samples, hybridization, staining, and scanning of the BeadChips were performed according to the Illumina Infinium II protocol at the array facility of ServiceXS (Leiden, the Netherlands). Data were analyzed using Illumina GenomeStudio software version 2009.2. The LogR ratio and the B allele frequency data were processed into the OverUnder plugin as described previously,²⁰ and copy numbers were calculated. Because of lack of normal tissue, it cannot be excluded that some of the copy number variation are germline polymorphisms.

WES

Genomic DNA was amplified using the illustra GenomiPhi V2 DNA amplification kit (GE Health Care, Little Chalfont, United Kingdom). Unamplified DNA of the B-LBL isolated from blood was used to control for amplification errors. Fragmentation was performed with the Covaris sonicator (GC Biotec, Alphen aan de Rijn, the Netherlands). Library preparation was performed using Solid chemistry. An exome capture was performed with whole-exome capture EZ version 3 of Nimblegen (Roche, Almere, the Netherlands) with platform-specific adaptors and blockers. Clonal amplification of the captured library was performed by emulsion PCR (Life Technologies, Carlsbad, CA). Finally, samples were sequenced on a Solid 5500 xl sequencer. Obtained data were processed according to the NGS pipeline described by Houten et al.²¹ Because of the lack of normal tissue, only differential variants between FL and B-LBL could be determined. In addition, in our WES data we also checked for mutations in sets of 92 and 40 genes, previously found to be mutated in WES data of FLs and B-cell acute lymphoblastic leukemias (B-ALLs), respectively (Supplemental Table S1).^{5,22–26} Eleven mutations, in 10 genes identified in TF3b, were validated by Sanger sequencing in TF3b, and, in addition, these 11 mutations were examined in TF1a, TF2a, and TF2c. Moreover, enhancer of zeste homolog 2 (EZH2), which is frequently mutated in FL, was screened for mutations in TF1a and TF3b.

Results

Histology and Phenotype of Four FLs before and after Transformation into B-LBLs

Before transformation, the four FLs (TF1a to TF4a) displayed a follicular growth pattern with a characteristic phenotype, that is, CD20⁺, CD79a⁺, BCL2⁺ cells co-expressing the GC markers CD10 and BCL6 that expanded in networks of follicular dendritic cells (Tables 1 and 2). After B-LBL transformation, the follicular architecture of TF1, TF2, and TF4 was lost. The tumor cells of all four cases still expressed CD79a, CD10, and BCL2 but were devoid of CD20. In addition, all B-LBLs expressed TdT. In the TF2b B-LBL approximately 10% of the tumor cells stained positive for TdT. However, no TdT expression was observed in the TF2c biopsy. In TF3b, the follicular growth pattern was in part retained and alternated with sheets of lymphoblastic cells (Figure 1). To analyze the molecular features of the entities in TF3b, CD19⁺ cells of lymph node suspensions underwent fluorescence-activated cell sorting based on size and expression of CD20 and CD10, in: i) small CD19⁺CD10⁺CD20^{lo} cells, ii) small CD19⁺CD10⁺CD20^{hi} cells, iii) small CD19⁺CD10⁻CD20^{hi} cells, and iv) large CD19⁺CD10⁺CD20^{lo} cells. Both CD20^{lo} fractions expressed TdT, as determined by RT-PCR, demonstrating that these fractions represent the B-LBL

Table 2 Immunophenotypes of Four Lymphomas before and after Progression

Patient	Diagnosis	CD20	FDC	CD10	BCL6	BCL2	TdT	Pax5	CD79a	CD19	Igλ	Igκ
TF1a	FL	+	+	+	+	+	—	+	+	+	+	—
TF1b	B-LBL	—	ND	+	—	+	+	+	+	+	+	—
TF2a	FL	+	+	+	+	+	—	+	+	ND	ND	ND
TF2b	B-LBL	—	ND	+	+	+	+	+	+	+	+	—
TF2c	B-LBL	—	ND	+	+	+	—	+	+	+	+	—
TF3a	FL	+	+	+	+	+	—	+	+	ND	ND	ND
TF3b	FL	+	+	+	+	+	—	+	+	+	ND	ND
	B-LBL	—	—	+	—	+	+	+	+	+	ND	ND
TF4a	FL	+	ND	+	+	+	—	+	+	+	ND	ND
TF4b	DLBCL	—	—	+	—	+	—	+	+	ND	ND	ND
TF4c	B-LBL	—	—	+	—	+	+	+	+	ND	ND	ND

Protein expression of CD20, CD21-L, CD10, BCL6, BCL2, TdT, Pax5, CD79a, CD19, and Igλ, as determined by IHC on formalin-fixed paraffin-embedded and frozen sections.

BCL, B-cell lymphoma; B-LBL, precursor B-cell lymphoblastic lymphoma; FDC, follicular dendritic cells (as determined by CD21-L staining); FL, follicular lymphoma; LN, lymph node; ND, not determined due to lack of appropriate patient material; TdT, terminal deoxynucleotidyl transferase.

(Supplemental Figure S1). Of note, the FL of TF4 transformed into a DLBCL before transforming into B-LBL (Table 1).

The Four FLs and Successive B-LBLs Express Clonally Related Somatically Mutated Ig γ Heavy Chains

IGHV gene analysis showed that in all patients the FL and B-LBL were of the same clonal origin, harboring identical IGHV rearrangements (Table 3). In TF3b, all four fractions undergoing fluorescence-activated cell sorting, including the small CD19⁺CD10[−]CD20^{hi} cells, harbored an identical IGHV rearrangement, suggesting that this population is either a CD10[−] subclone of the FL or represents a stage intermediate between the FL and B-LBL. In accordance with their GC phenotype, all four FLs harbored somatically mutated IGHV. Intriguingly, all four

lymphomas expressed IgG, as determined by RT-PCR, instead of the commonly found IgM in FL⁴ (Table 3). Because of the poor DNA quality, the IGHV of the *TF3a* FL could only be investigated from framework 2. We were unable to determine the IGHV of the TF4b DLBCL due to lack of material.

All four FL/B-LBL pairs harbored high numbers of shared somatic IGHV mutations (Figure 2), further underscoring their clonal relationship. Comparison of individual molecular IGHV clones of TF1 and TF3 showed intraclonal IGHV sequence variation of the FLs but not of the B-LBLs. Particularly the CD20^{hi} FL cell fractions of TF3b showed high intraclonal IGHV sequence variation, whereas intraclonal IGHV sequence variation was not detected in the CD20^{lo} B-LBL cell fractions (Figure 2).

Figure 1 Histology and immunophenotype of TF3 before and after transformation. 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 (as indicated by an **arrowhead**), which was alternated by sheets of lymphoblastic cells (as indicated by an **arrow**). The FL cells still expressed CD20, BCL6, and BCL2, whereas the B-LBL cells lacked expression of CD20 and BCL6 but expressed TdT. Original magnification, ×100. BCL, B-cell lymphoma; B-LBL, B-lymphoblastic lymphoma; FL, follicular lymphoma; TdT, terminal deoxynucleotidyl transferase.

Table 3 IGHV and IGLV Rearrangements of the Four FLs and in Their Ensuing B-LBL

Patient	Diagnosis	Subclass	IGHV rearrangement	Mut no. (shared with FL)	IGLV rearrangement
TF1a	FL	IgG	IGHV4-61/NA/IGHJ5a	22	IGLV2-18/IGLJ2/3a
TF1b	B-LBL	IgG	IGHV4-61/NA/IGHJ5a	27 (19)	IGLV3-21/IGLJ3b
TF2a	FL	IgG	IGHV3-20/NA/IGHJ6b	23	IGLV1-36/IGLJ3b
TF2b	B-LBL	IgG	IGHV3-20/NA/IGHJ6b	23 (23)	Polyclonal
TF3a	FL	ND	IGHV1-69/IGHD1-26/IGHJ6c	10*	ND
TF3b [†]	FL (CD10 ^{hi})	IgG	IGHV1-69/IGHD1-26/IGHJ6c	17	IGLV4-69/IGLJ2/3a
	FL (CD10 ^{low})	IgG	IGHV1-69/IGHD1-26/IGHJ6c	15	IGLV4-69/IGLJ2/3a
	B-LBL (S)	IgG	IGHV1-69/IGHD1-26/IGHJ6c	15 (14)	IGLV4-69/IGLJ2/3a
	B-LBL (L)	IgG	IGHV1-69/IGHD1-26/IGHJ6c	15 (14)	IGLV4-69/IGLJ2/3a
TF4a	FL	IgG	IGHV3-73/IGHD1-7/IGHJ5*02	21	ND
TF4b	DLBCL	ND	ND	ND	ND
TF4c	B-LBL	IgG	IGHV3-73/NA/IGHJ5*02	25 (18)	ND

*IGHV SHM was determined starting from FR1 in all instances, except for TF3a, in which SHMs were determined starting from FR2, and TF4, in which SHM could not be determined in the first part of FR1.

[†]Lymph node suspensions of TF3b were sorted in four fractions (Supplemental Figure S1 and text).

B-LBL, precursor B-cell lymphoblastic lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; FR, framework; L, large; Mut, mutation; NA, not applicable; ND, not determined; S, small; SHM, somatic hypermutation.

Some B-LBLs Show Ongoing Light Chain Rearrangement and Express Surrogate Light Chains

The FL TF1a was found to express an IGLV2-18/IGLJ2/3a rearrangement (Table 3). Interestingly, after transformation a different monoclonal Ig λ variable region (IGLV) rearrangement was amplified out of the TF1b B-LBL, that is, IGLV3-21/IGLJ3b. Amplification of IGLV of DNA followed by GeneScan analysis revealed that in both the FL and the B-LBL of TF1 two IGLV rearrangements were present (data not shown). One of these IGLV rearrangements was of the same length before and after transformation. In accordance with the IGLV sequence analysis, the second IGLV rearrangement in the B-LBL of TF1b was of a different length than that of the FL of TF1a. The IGLV3-21 gene segment, identified in the B-LBL, is located more upstream in the IGLV locus than the IGLV2-18 gene segment of the FL. Most likely, the IGLV3-21 rearrangement of the TF1b B-LBL has emerged by secondary rearrangement of the IGLV. Similarly, the IGLV1-36/IGLJ3b rearrangement detected in FL TF2a was not found in B-LBL TF2b. In TF2b an oligoclonal rearrangement pattern was seen by GeneScan analysis (data not shown). In all cell populations of TF3b undergoing fluorescence-activated cell sorting, an identical IGLV4-69/IGLJ2/3a rearrangement was observed. In accordance, a monoclonal IGLV region of identical length was observed by GeneScan analyses (data not shown).

The expression of the Ig light chain was also assessed by immunohistochemistry (IHC) on cryostat sections. The tumor cells of both the FL and B-LBL of TF1 expressed Ig λ , but no Ig κ (Table 2), confirming the RT-PCR analyses. In the B-LBL TF2b, Ig λ was exclusively detected. IGLV expression of FL TF2a and all TF3 and TF4 samples could not be investigated by IHC due to lack of frozen tissue.

It has been described that B-LBLs express the surrogate light chain (ψ L), which is composed of two proteins, VpreB and λ 5.²⁷ RT-PCR analyses revealed that both the B-LBL of TF1b and TF2b indeed expressed VpreB and λ 5, whereas the preceding FL of TF1a and TF2a did not (Figure 3). In TF3a and TF3b, VpreB and λ 5 expression were not detected. Because of the lack of material, VpreB and λ 5 expression was not assessed in TF4.

All Four B-LBLs Acquired a MYC Gene Translocation

In accordance with the immunohistochemical data, the FLs of TF1a, TF3a, and TF4a harbored translocations of the *BCL2* gene as detected by segregation FISH, and these translocations were also detected after histologic transformation. Unfortunately, the *BCL2* FISH on TF2a was not interpretable; however, in the TF2c B-LBL a *BCL2* translocation was detected (Table 4). In all four patients, *MYC* gene translocations were identified after histologic transformation. In addition, a 3q27 break, involving *BCL6*, was found in the FL of TF1a, whereas after progression no split signals were detected. In contrast, the B-LBL of TF2c showed a 3q27 break only after transformation. In TF3 and TF4, a 3q27 breakage was not detected in any of the biopsies. Both in TF3 and TF4, *BCL6* protein expression decreased on transformation, as detected by IHC (Figure 1).

Genome-Wide Analyses of TF3 before and after Transformation

To further uncover the genomic aberrations that accompanied progression of the FL of TF3, the sorted cell populations were analyzed by comparative genomic hybridization (CGH). The number of genomic aberrations in the FL of TF3b was similar to two control FLs. In total, the FL and B-LBL fractions of TF3b shared 26 genomic

Figure 2 IGHV configurations of FL and B-LBL. Schematic representation of IGHV consensus sequences of TF1 to TF4 before and after progression. **Vertical bars** correspond to shared mutations between the FL and B-LBL. V corresponds to two shared mutations in the same codon. Nonshared replacement and silent mutations are indicated by closed and open lollipop symbols, respectively. Codon numbering is according to V-Quest version 3.3 (http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanIg).¹⁸ Black boxes indicate parts of the IGHV gene that were not sequenced. B-LBL, B-lymphoblastic lymphoma; CDR, complementarity-determining region; FL, follicular lymphoma; ICV, degree of intraclonal variation; L, large; ND, not determined; S, small.

aberrations (Figure 4A), underscoring their clonal relationship. The shared aberrations comprised three amplifications, three deletions, and copy-neutral loss of heterozygosity (CN-LOH) in 20 regions, including deletion of 22q11 and CN-LOH of 6q21 and 7q33, which are all commonly observed in FLs²⁸ (Table 5). Interestingly, these 26 shared aberrations also contained regions previously described to be associated with transformation of FL into high-grade lymphomas other than B-LBL, such as deletion of 1p36.²⁸ It was noted by CGH that *MYC* was amplified in the FL before translocation (Figure 4A). The B-LBL harbored only a limited set of aberrations in addition to the aberrations

already present in the FL, that is, CN-LOH of 9p, amplification of 14q32, and deletion of 17q12. The affected region of 9p included CN-LOH of *JAK2* and *PAX5*. Importantly, a small region of 9p21 was deleted, which contains the tumor suppressor genes *CDKN2A/B* (encoding p15 and p16) and *MTAP* (Figure 4B). Genes amplified at 14q32 include the proto-oncogenes *TCL1*, *BCL11*, and *AKT1* (*PKB*). The tumor suppressor gene *NF-1* is among the genes deleted on chromosome 17. In Supplemental Table S2, the affected genes in 9p, 14q32, and 17q12 are listed. In the CD10⁺ fraction, an amplification of chromosome 12 was gained compared with the FL fraction. It did however not share any

Figure 3 VpreB and λ5 are expressed in the B-LBL but not in the FL. RT-PCR analysis of *VpreB* and *Lambda5* expression before and after progression. TF3 was sorted in four fractions (Supplemental Figure S1 and text). The - indicates a negative water control and the pre-B-cell line Nalm-6 was used as a positive control (+). B2M, β-2-microglobulin; B-LBL, B-lymphoblastic lymphoma; FL, follicular lymphoma; L, large; S, small.

Table 4 Chromosomal Translocations Detected by FISH Analysis

Patient	BCL2	MYC	BCL6
TF1a	+	—	+
TF1b	+	+	—
TF2a	NI	—	—
TF2c	+	+	+
TF3a	+	—	—
TF3b	+	+	—
TF4a	+	—	—
TF4b	+	—	ND
TF4c	+	+	—

BCL, B-cell lymphoma; FISH, fluorescence *in situ* hybridization; ND, not determined; NI, not interpretable.

of the aberrations found in the transformed B-LBL, suggesting that this fraction is a subclone of the FL rather than a tumor population at an intermediate stage between FL and B-LBL.

WES revealed heterozygous missense mutations in *CCND3*, *DYSF*, *ISOC2*, and *ZFHX4* present in the B-LBL, which were absent in the FL. Two missense mutations (in *SMARCA2* and *SNAPC3*) heterozygously present in the FL were hemizygous in the B-LBL due to LOH of chromosome 9p. In addition, one nonsense mutation in *TNFRSF14* and additional mutations in the 5' untranslated region of *BCL2* and *MYC* were found in the B-LBL (Table 6 and Supplemental Figure S2). The mutations in *CCND3*, *ISOC2*, *MYC*, and *ZFHX4* were found in 7%, 9%, 3%, and 6% of the sequence reads, respectively, and thus were subclonal variants in the FL. Next to this analysis, we checked a set of genes previously described to be mutated in FL and B-ALL (see Materials and Methods). This unveiled missense mutations in *FMN2*, *NEB*, and *SYNE1*, as well as a nonsense mutation in *KMT2D*, were shared by the FL and B-LBL. In addition, 1 synonymous, 2 3' untranslated region, and 14 intronic mutations were identified (Supplemental Table S2). Eleven mutations in 10 genes were validated by Sanger sequencing in TF3b (Supplemental Figure S2). These mutations were also examined in TF1a, TF2a, and TF2c but were however not detected (data not shown). Moreover, no mutations in the commonly mutated gene *EZH2* were detected in TF1a and TF3b. The loss of *CDKN2A/B* as detected by CGH was confirmed by comparing the numbers of sequence reads (data not shown).

Discussion

Transformation of FL into a B-LBL occurs rarely and has been documented for a limited number of cases.^{7–15} Here, we describe four B-LBLs that evolved out of FLs and show that, despite retained expression of mutated IGHV genes, the B-LBLs had acquired several key features of precursor B cells. The follicular architecture as well as CD20 expression were lost; the expression of TdT, VpreB, and Lambda5 was induced; and evidence was obtained for

reinitiated IGLV gene rearrangement because the expressed IGLV genes in the B-LBL of TF1b and TF2b were different from those of the FL of TF1a and TF2a, respectively, whereas the IGHV genes were unaltered.

A t(14;18)(q32;q21) is a rare event in primary B-LBL/B-ALL. Stamatoullas et al²⁹ reported that of 142 adult B-ALLs, only 5 had a translocation involving the *BCL2* gene. The Mitelman database (<http://cgap.nci.nih.gov/Chromosomes/Mitelman>, last accessed June 30, 2016) contains 12 *de novo* B-LBLs that harbor both a *BCL2* and a *MYC* translocation. In the studies assembled in the Mitelman database no IGHV sequence analyses were performed; thus, it is not excluded that the t(14;18)⁺ B-LBLs include cases that originate from FL.

Here, in all four lymphomas a *MYC* translocation was gained on histologic transformation. Secondary *MYC* translocations have been reported by others in 18 of 19 FLs transforming into B-LBLs,^{7–15} suggesting that *MYC* overexpression is mandatory for transformation into a B-LBL. However, this event by itself is unlikely to impose an immature B-cell program, given that 10% of FLs transforming into a DLBCL also carry *MYC* rearrangements.³⁰ Remarkably, in the FL of TF3, *MYC* was amplified before translocation as determined by CGH. It has been reported in mice that *MYC* translocation is promoted by activation-induced cytidine deaminase (AID) activity.³¹ In this respect, the observations that the FLs of TF2 and TF3 display intracлонаl IGHV sequence variation and that all four FLs express IgG, whereas most FLs express IgM,² are noteworthy and likely to be AID mediated. However, by RT-PCR we detected no AID expression in TF1, TF2, or TF3 (data not shown). Interestingly, IgG expression was also noticed in two separately reported FLs that transformed into B-LBLs.^{13,14}

In TF2, progression to B-LBL was accompanied by a break at 3q27. Similarly, *BCL6* translocations have also been documented in other *de novo* t(14;18)⁺ B-LBLs.²⁹ *BCL6* region translocations are known to occur in 6% to 10% of (grade 3) FLs,³² approximately 30% of DLBCLs,³² and approximately 10% of FLs transforming into DLBCL.³³ TF2b is a so-called triple-hit lymphoma carrying rearrangements of the *BCL2*, *BCL6*, and *MYC* genes. This combination of genomic rearrangements has been reported before in sporadic cases of FL undergoing transformation to DLBCLs as well as in *de novo* DLBCLs.^{6,34,35} Both the t(14;18)(q32;q21) and the t(8;14)(q22;q32) disrupt the *IG* coding region.^{36,37} However, the finding that in TF2b IGHV mRNA is still expressed is not necessarily contradictory because *BCL6* locus rearrangements may engage the *IGL* chain or non-*IG* loci, whereas *MYC* may be juxtaposed to downstream *IG* switch regions, thus leaving the *IG* coding region intact.³⁸ Alternatively, *BCL2* and *MYC* rearrangements may occur at the same *IGH* allele.³⁹ The phenomenon that in TF1 the break at the 3q27 locus was no longer detected after progression into the B-LBL of TF1b has been reported in FLs transformed to DLBCL.⁴⁰

Figure 4 Genomic aberrations of TF3b FL and B-LBL as determined by comparative genomic hybridization. **A:** Overview of genomic aberrations of the four sorted cell populations of TF3b and two control FLs, as determined by comparative genomic hybridization. Red indicates homozygous deletion. Orange indicates heterozygous deletion. Green indicates CN-LOH. Blue indicates twofold amplification. Purple indicates greater than threefold amplification. **B:** Allele frequency of chromosome 9p in the B-LBL of TF3b. The green area indicates CN-LOH. Red indicates deletion. B-LBL, B-lymphoblastic lymphoma; Chr, chromosome; CN-LOH, copy-neutral loss of heterozygosity; FL, follicular lymphoma; Freq, frequency.

High-resolution CGH array on sorted FL and B-LBL cells from TF3b revealed that the B-LBL had gained only a limited number of genetic aberrations, that is, CN-LOH of 9p; deletion of 17q12; amplification of the oncogenes *TCL1*,

BCL11B, and *AKT*, all on chromosome 14; and amplification of 14q32 which includes the gene encoding the transcription factor Yin Yang 1 (*YY1*). *YY1* is expressed in early B cells and GC B cells, where it regulates

Table 5 Numerical Genomic Aberrations in TF3

Region	FL	B-LBL	Remarks
1p36	Del.	Del.	Associated with transformation of FL
2p16	CN-LOH	CN-LOH	Ampl. associated with transformation of FL
5p12	CN-LOH	CN-LOH	Ampl. associated with transformation of FL
6q21	CN-LOH	CN-LOH	Del. in 17% of FL ²⁸
6q22	CN-LOH	CN-LOH	Ampl. associated with transformation of FL
7q33	CN-LOH	CN-LOH	LOH in 11% of FL ²⁸
8q	Ampl.	Ampl.	Includes ampl. of <i>MYC</i>
9p	WT	CN-LOH	Includes del. of <i>CDKN2</i>
14q32	WT	Ampl.	Includes ampl. of <i>TCL1</i> and <i>BCL11</i>
17q12	WT	Del.	Includes del. of <i>NF-1</i>
22q11	Del.	Del.	Del. in 10% of FL ²⁸

Ampl., amplification; B-LBL, precursor B-cell lymphoblastic lymphoma; CN-LOH, copy neutral loss of heterozygosity; Del., deletion; FL, follicular lymphoma; WT, wild-type.

IGHV-rearrangement and class switch recombination, respectively.⁴¹ YY1 has also been shown to activate the *MYC* promoter.⁴² The deleted 9p region contains *CDKN2A/B* and *MTAP* and was previously described to be associated with transformation of FL into DLBCL.⁵ Loss of *CDKN2A/B* also occurs in approximately 40% of adult B-ALLs and is associated with poor survival.⁴³ Deletion of the cell cycle regulators *CDKN2A/B* may be required for transformation because it renders the cells resistant to *MYC*-induced apoptosis.⁵ It is, however, unlikely that *CDKN2A/B* loss is instrumental in the induction of a precursor B-cell phenotype.

PAX5, a master regulator of B-cell lineage commitment and differentiation⁴⁴ is among the genes on 9p that show LOH after transformation (listed in Supplemental Table S2). Interestingly, mutations in *PAX5* were found in approximately 30% of *de novo* B-LBLs, suggesting that loss of expression of this transcription factor results in a developmental blockade and drives B-LBL development.⁴⁴

Moreover, it was shown that *PAX5*^{-/-} mice have a differentiation arrest at the pro-B-cell stage,⁴⁴ whereas targeted deletion of *PAX5* in mature B cells resulted in loss of the B-cell phenotype and lymphoblast formation.⁴⁵ However, we did not detect mutations in the coding region of *PAX5* in TF1, TF2, and TF3 (data not shown), and *PAX5* protein expression was measurable by IHC in all four B-LBLs (Table 2). Still, decreased *PAX5* transcription and protein expression due to the 9p deletion cannot be excluded. Neither did we detect mutations in *IKZF1* in TF3 (data not shown), another master regulator of B-cell commitment and differentiation, which is frequently deleted in *de novo* B-LBL.⁴⁴

WES revealed missense mutations in *FMN2*, *NEB*, and *SYNE1* as well as a nonsense mutation in *KMT2D*, shared by the FL (TF3a) and B-LBL (TF3b) (Supplemental Table S1). These genes were previously described to be mutated in FL.^{5,22–26} *KMT2D* has a role in epigenetic programming and is considered as a driver gene of FL.²² Compared with the preceding FL (TF3a), seven missense mutations and one nonsense mutation were gained in the B-LBL stage. One of the missense mutations in *CCND3* (T283A) is located in exon 5, a region recurrently mutated in DLBCL and BL. In 38% of sporadic BL cases a mutation in *CCND3* is present.⁴⁶ The T283A mutation was shown to increase protein stability of *CCND3* conferring a proliferative advantage.⁴⁶ In the FL stage, a deletion of chromosome 1p36 was detected, including deletion of *TNFRSF14*. In FL, –1p36 is found in frequencies between 20% and 40% and has been correlated with dismal prognosis.^{28,47} Interestingly, in addition to –1p36, in the TF3b B-LBL a nonsense mutation in *TNFRSF14* was found, suggestive for a tumor suppressor role in the progression of FL. However, there are conflicting reports as to whether the combination of –1p36 with *TNFRSF14* mutation is associated with good or bad overall survival.^{48,49} Interestingly, the mutation in *SMARCA2* (*BRM*), which is part of the switch/sucrose non-fermentable ATP-dependent chromatin remodeling complex, was hemizygotously found in the B-LBL stage due to LOH on chromosome 9p.⁵⁰ Mutations in *SMARCA2* and

Table 6 Exome Mutations Gained in the B-LBL of TF3b

Gene	Chromosome	Position	Mutation type	RefSeq	Mutation	FL	B-LBL
<i>BCL2</i>	18	60986079	5' UTR	NM_00633	5' UTR-180 C/T	WT	Het
<i>CCND3</i>	6	41903710	Missense	NM_001760	K268R (A803G)	WT	Het
		41903754	Missense		T283A (A847G)	WT	Het
<i>DYSF</i>	2	71908237	Missense	NM_01130987	P2057Q (C6170A)	WT	Het
<i>ISOC2</i>	19	55967760	Missense	NM_01136201	G94A (A32T)	WT	Het
<i>MYC</i>	8	128748820	5' UTR	NM_002467	5' UTR+19 T/C	WT	Het
<i>SMARCA2</i>	9	2081868	Missense	NM_003070	N741H (A2221C)	Het	Hem
<i>SNAPC3</i>	9	15451376	Missense	NM_001039697	T264S (A791C)	Het	Hem
<i>TNFRSF14</i>	1	2488138	Nonsense	NM_003820	W12* (G35A)	WT	Hom
<i>ZFXH4</i>	8	77763412	Missense	NM_024721	H1419Y (C4255T)	WT	Het

B-LBL, precursor B-cell lymphoblastic lymphoma; FL, follicular lymphoma; Hem, hemizygous; Het, heterozygous; Hom, homozygous; UTR, untranslated region; WT, wild-type.

SMARCA4 were described in marginal zone B-cell lymphoma,⁵⁰ BL, and DLBCL.⁴⁶ *SMARCA2* is a known interaction partner of C/EBP α , which forms heterodimers with C/EBP β .⁵¹ It has been shown that C/EBP α and C/EBP β are involved in B-cell reprogramming to macrophage-like cells or stem cell-like cells. However, it is unclear whether *SMARCA2* interacts with the heterodimer of C/EBP α and C/EBP β and whether this interaction is necessary for a role in B-cell reprogramming.⁵² In addition, switch/sucrose non-fermentable complex components, including *SMARCA2*, are necessary for the accessibility of the *CD79A* promotor for the transcription factors PAX5 and EBF1.⁵³ Via these mechanisms the identified mutation in *SMARCA2* may contribute to the lymphoma de-differentiation. Some of the mutations that were specifically and uniformly detected in the B-LBL (in *CCND3*, *ISOC2*, *MYC*, and *ZFHX4*) were detectable in 3% to 9% of the sequence reads in the preceding FL time point. This observation indicates that minor precursor clones containing these mutations were present in the FL. One subclone of the FL with all these mutations eventually grew out as the B-LBL.

In a recently published study by Geyer et al¹² seven FLs, which transformed into B-LBLs, were analyzed. Similar to our study, all transformation cases were characterized by loss of follicular architecture, gain of TdT expression, and the presence of a *MYC* gene rearrangement. The lymphoblastic lymphoma of one case was analyzed by WES also identifying mutations in *TNFRSF14* and *CCND3*. Unfortunately, it is unknown whether these mutations had been present in the FL, because the preceding FL was not analyzed.

Some of the genomic mutations acquired in the B-LBL are also implicated in the transformation from FL to DLBCL, such as deletion of *CDKN2A/B* and *MYC* translocation. *MYC* translocation is also essential in BL development, and the acquired *CCND3* mutation (T283A) is frequently mutated in this aggressive lymphoma. Although we also determined several acquired mutations, which were not described in either DLBCL or BL and intriguingly all FLs transforming to B-LBL expressed IgG, we cannot explain this peculiar transformation.

It was previously hypothesized that clonally related FLs and B-LBLs do not develop sequentially but might evolve in parallel from precursor cells harboring the t(14;18).^{8,36} A t(14;18)⁺ sIg⁻ and TdT⁺ B cell might thus evolve into a TdT⁻CD20⁺ FL or expand as a precursor B cell by acquisition of an additional *MYC* translocation. However, more recent work has shown that after the acquisition of the t(14;18) in bone marrow precursor B cells, these t(14;18)⁺ B cells further mature to memory B cells and acquire somatic hypermutations in successive rounds of re-entry in germinal centers.⁵⁴ Thus, the B-LBL might originate from mature postgerminal center B cells functioning as FL precursor cells that undergo a further round of re-entry in a germinal center, thereby acquiring an AID-induced *MYC*

breakpoint. For still unknown reasons they also show partial regression to a phenotype of precursor B cells. Our study, however, demonstrates that the B-LBLs evolve from FL subclones that already accumulated most (for TF2 all) of the somatic IGHV mutations and for TF3 also most of the genomic aberrations. Whatever the exact mechanism may be, these data clearly show that the FL and B-LBL pairs did not arise in parallel from t(14;18)⁺ Ig unmutated precursor cells.

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Supplemental Data

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