Cytogenetic Alterations Affecting *BCL6* Are Predominantly Found in Follicular Lymphomas Grade 3B with a Diffuse Large B-Cell Component

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Recently, classical banding cytogenetic studies suggested that follicular lymphomas (FLs) grade 3 with preserved maturation to centrocytes (FL3A) are closely related to FL grades 1 and 2 and frequently harbor the t(14;18), whereas FL grade 3B, consisting of centroblasts exclusively, do frequently show 3q27 alterations. To clarify the prevalence of *BCL6* and *BCL2* rearrangements in FL and diffuse large B-cell lymphomas (DLBLs), we performed a large scale bicolor interphase cytogenetic (fluorescence *in situ* hybridization) study on 188 well-characterized B-NHLs classified according to the World Health Organization Classification of Tumors of the Lymphoid Tissues. *BCL6* rearrangements were detected in a significantly higher number of FL3B with a DLBL component (12 of 22, 55%) compared with purely diffuse nodal DLBLs (19 of 77, 25%) and DLBLs with a well-documented primary extranodal origin (2 of 27, 7%) ($P < 0.001$). Five FL3B without a DLBL component were negative for both t(14;18) and 3q27 aberrations. FL grades 1/2 and FL3A were t(14;18)-positive in 88% and 64% of cases, respectively, but 3q27 alterations were identified in only four FL3A. These data exemplify different genetic pathways in the genesis of FLs with a high content of centroblasts and suggest that 3q27 rearrangements are predominantly associated with FL grade 3B harboring a DLBL component. (*Am J Pathol* 2004, 165:481–490)

Follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBL) represent the two most common entities of non-Hodgkin's lymphoma (NHL) worldwide with up to 22% and 31% of tumors, respectively.^{1,2} FLs have been classically associated with the translocation t(14;18)(q32;q21) leading to the rearrangement of the *BCL2* gene in 70 to 95% of reported tumors, whereas translocations involving the *BCL6* proto-oncogene locus in chromosomal band 3q27, like the t(3;14)(q27;q32), have been described in DLBLs in up to 40% of cases. There is some overlap in genetic features, however, with rearrangements of *BCL6* encountered in 6 to 14% of FLs and of *BCL2* in 20 to 30% of DLBLs.³

The new World Health Organization Classification of Tumors of the Hematopoietic and Lymphoid Tissues takes into account the different morphological appearance of FL grade 3. FL3A are composed of centroblasts and centrocytes, whereas FL3B consist of centroblasts exclusively.³ Recently, we have been able to dissect FL grade 3 in one type of FL3 harboring the t(14;18) in more than 70% (FL3A) and one type of FL3 composed of centroblasts (FL3B) and frequently in combination with a DLBL component (FL3B+DLBL) associated with a *BCL6* gene rearrangement in ~40%.⁴ Because the t(3;14)/3q27 rearrangements have been classically associated with DLBL, we wished to precisely assess the prevalence of *BCL6* rearrangements in FL and DLBL. Therefore, we performed a large scale investigation on 188 nodal and extranodal B-cell NHLs using bicolor interphase fluorescence *in situ* hybridization (FISH), a powerful tool to detect rearrangements affecting oncogene loci in B-NHL.^{5,6}

In this work, FL3B+DLBL were shown to harbor 3q27/*BCL6* alterations significantly more often than FL3A and purely diffuse nodal and extranodal LBL ($P < 0.001$) indicating different genetic pathways in the development of the two types of FL3 and that tumors characterized by 3q27 rearrangements predominantly present with centroblastic cytology and a partially follicular growth pattern. Because of their frequent implication of the *p16^{INK4a}*, *TP53*, and *D13S25* gene loci in the progression of FL,^{7–12} we also performed FISH for the status of these loci.

Materials and Methods

Tumor Specimens

One hundred eighty-eight nodal and extranodal B-cell NHLs, referred to the Department of Pathology at the University of Würzburg between 1987 and 2002, were classified according to the criteria of the World Health Organization classification system.³ Classification of a tumor as follicular or partially follicular required a follicular growth pattern in at least 25% proven by the presence of

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follicular dendritic cell meshworks. Routine histology was performed on slides cut from formalin-fixed and paraffin-embedded tissues using hematoxylin and eosin, Giemsa, periodic acid-Schiff, and Gomori's silver stainings. In 150 of 188 (80%) cases the tumor specimens were obtained before treatment and represented primary tumor biopsies.

Immunohistochemistry

Paraffin sections were immunostained for diagnostic purposes using antibodies directed against CD5 (clone 4C7, dilution 1:40; Novocastra, Newcastle on Tyne, UK), CD20 (clone L26, 1:1000; DAKO, Glostrup, Denmark), CD23 (clone 1B12, 1:10; Novocastra), and/or CD21 (clone 1F8, 1:20; DAKO), CD30 (clone Ber-H2, 1:10; DAKO), and Ki67 (MM1, 1:30; Novocastra). The expression of BCL-2, BCL-6, and CD10 was evaluated using monoclonal antibodies against BCL-2 (clone 124, 1:50; DAKO), BCL-6 (clone PG-B6p, 1:5; DAKO), and CD10 (NCL-CD10-270, 1:5; Novocastra). Although a cutoff level of at least 50% of the tumor cells was initially set up to regard a case as BCL-2- or CD10-positive, staining tended to show variations in the intensity of staining rather than variations in the number of positive cells. Therefore, in the vast majority of cases, tumors tended to be either positive (that is, distinctly greater than background) or negative. BCL-6 expression was recorded in well-fixed areas with the highest staining intensity in steps of 10%. In this study, we used high BCL-6 reactivity ($\geq 70\%$ cells with nuclear staining) as a cutoff, being approximately equivalent to the percentage of BCL-6-positive cells encountered in nonneoplastic follicle centers. To detect p53 overexpression on paraffin sections, the DO-7 antibody (1:50, DAKO) was applied and regarded as positive if more than 20% of the cells showed strong nuclear expression.

Monotypic cytoplasmic reactivity for κ - or λ -immunoglobulin light chains (Clg+) was assessed using rabbit anti-human λ and κ light chain antibodies (1:40,000 and 1:20,000; DAKO). All immunohistochemical reactions were accomplished by a modified peroxidase anti-peroxidase (PAP) technique using diaminobenzidine as chromogen after antigen retrieval by pressure cooking as previously described by Rüdiger and colleagues.¹³

Bicolor FISH Analyses

To obtain well-preserved and separately located mononuclear cells for interphase cytogenetic analysis, methanol:acetic acid (3:1) fixed cell suspension from cytogenetic preparations or from mechanically disaggregated fresh frozen tissues (Medimachine, DAKO) were dropped onto glass slides. The prevalence of *BCL6* rearrangements was assessed in all cases by bicolor interphase FISH using a split apart locus-specific probe flanking the *BCL6* breakpoint at 3q27 (Vysis, Stuttgart, Germany) following the manufacturer's advice (Figure 1f). Detection of the translocation t(14;18)(q21;q32) (Figure 1e) and deletions of *TP53*, *D13S25*, and *P16*^{INK4a} loci were done using respective probes (Vysis). Because classical banding analyses and FISH revealed no different results regard-

ing the translocation t(14;18)(q32;q21) in a series of 85 cases [including 20 t(14;18)-positive and 65 negative lymphomas, among them 3 FL1/2 and 2 FL3A cytogenetically negative for the t(14;18)], additional FISH analyses were done only in cases of no available cytogenetic data ($n = 32$). At least 100 intact nuclei per case were evaluated and an aberrant clone was defined according to the cutoff level for each probe evaluated in control studies with five reactive lymph node specimens by the mean percentage of cells with aberrant signals in at least 200 cells plus three standard deviations (*BCL6*, 6%; t(14;18)(q32;q21), 5%; *TP53*, 5%; *D13S25*, 5%; and *P16*^{INK4a}, 5%). Signal visualization was accomplished by a Zeiss Axioskop2 fluorescence microscope (Zeiss, Jena, Germany) and illustrations were made using the ISIS imaging system (MetaSystems, Altlusheim, Germany).

Cytogenetic Studies

Classical cytogenetic data were available in 156 of 188 (83%) cases investigated and had been obtained according to standard protocols.¹⁴ A part of the cytogenetic results was published earlier.^{4,14} Metaphases were evaluated according to the guidelines of the International System for Human Cytogenetic Nomenclature (ISCN).¹⁵ Briefly, 10 ml of unstimulated cell suspension with 1 to 2×10^6 cells per ml of RPMI 1640 medium were set up and directly processed or allowed to grow overnight. Then the cultures were pulsed with colchicine for 30 minutes before harvesting. For metaphase preparation the cells were exposed to hypotonic solution of 0.075 mol/L KCL, fixed in methanol/acetic glacial acid (3:1), and after several washing steps, dropped onto glass slides. After maturation of the preparations for several days at room temperature, metaphases were stained using a modified trypsin-Giemsa technique. Images were captured with a Zeiss Axioskop 2 microscope (Zeiss) and evaluated using the IKAROS imaging system (MetaSystems). A chromosomal aberration was regarded as clonal, if either two or more metaphases of one case harbored the same structural alteration or chromosomal gain, or if a loss of one chromosome was found in at least three different metaphases.

Molecular Analyses for *BCL6* Rearrangements

High-molecular weight DNA was extracted from frozen tissues of 52 of 188 (28%) nodal and extranodal B-cell NHLs according to standard methods and digested with appropriate restriction enzymes (*Bam*HI and *Xba*I). Each tissue block was controlled for the presence of representative tumor masses before DNA extraction. After electrophoresis on 0.7% agarose gels, Southern blotting was performed on nitrocellulose filters. The filters were hybridized with a ³²P random-labeled DNA probe: a 4-kb *Sac*I fragment of the *BCL6* gene, generous gift of R. Dalla-Favera (Institute for Cancer Genetics, Columbia University, New York, NY).¹⁶ Cytogenetic data were available from 44 of 52 (85%) of these cases and FISH for 3q27 alterations was performed in all specimens (Table 4).

Figure 1. FL grade 3A (**a**, **c**, **e**) and FL grade 3B (**b**, **d**, **f**). **a:** Low-power magnification of FL3A illustrating indistinct germinal center borders with extensive interfollicular infiltration and merging (H&E). **b:** In contrast, follicles in FL grade 3B are sometimes sharply demarcated to the interfollicular T-zone (H&E). **c:** High magnification of FL3A illustrates many centroblasts and also sometimes enlarged centrocytes with more irregular nuclear contours (H&E). **d:** The cellular composition in FL3B is more homogeneous with some size variation in blasts yet centrocytes are absent (H&E). **e** and **f:** FISH in interphase nuclei: **e:** Detection of the t(14;18)(q32;q21) in the FL3A using dual-color fusion translocation probes by juxtaposition of the IgH locus in chromosomal band 14q32 (green signal) and *BCL2* in 18q21 (red signal) leading to a fusion signal. The two separated signals represent the nonrearranged chromosomes 14 and 18. **f:** Detection of *BCL6* alterations in the FL3B+DLBL using dual-color break apart probes by separating the green from the red signal localized centromeric and distal to the *BCL6* locus in band 3q27. The co-localized red and green signals represent the unrearranged chromosome 3. Original magnifications: $\times 200$ (**a**, **b**); $\times 1000$ (**c**, **d**).

Statistical Analysis

Statistical correlations were performed using chi-square and Mann-Whitney tests, respectively, depending on the nature of data. A *P* value less than 0.05 was regarded as statistically significant.

Results

Morphology and Classification

The classification of the 188 B-NHLs enrolled in this study is given in Table 1. Altogether, 74 (39%) lymphomas

Table 1. Morphological and Immunohistochemical Findings in 188 B-NHLs Enrolled in the Study

Diagnosis (n = 188)	CD10	BCL-2	BCL-6 ≥70%	p53 >20%	Clg+
FL1/2 (n = 24)	24/24 (100%)	24/24 (100%)	24/24 (100%)	0/24 (0%)	3/24 (13%)
FL3A (n = 23)	22/23 (96%)	20/23 (87%)	19/22 (86%)	3/23 (13%)	9/22 (41%)
FL3B (n = 5)	3/5 (60%)	3/5 (60%)	3/5 (60%)	0/5 (0%)	5/5 (100%)
FL3B + DLBL (n = 22)	7/22 (32%)	19/22 (86%)	9/22 (41%)	8/22 (36%)	14/22 (64%)
FL transformed (n = 10) (n = 10)	5/10 (50%)	9/10 (90%)	6/9 (67%)	4/10 (40%)	2/9 (22%)
DLBL nodal (n = 77)	24/77 (31%)	60/77 (78%)	33/76 (43%)	15/77 (19%)	42/77 (55%)
DLBL extranodal (n = 27)	9/27 (33%)	14/27 (52%)	9/26 (35%)	9/27 (33%)	12/27 (44%)
p FL1/2 versus FL3A	n.s.	n.s.	n.s.	n.s.	<.05
p FL3A versus FL3B ± DLBL	<.001	n.s.	<.001	n.s.	<.05
p FL1-3A versus FL3B ± DLBL	<.001	n.s.	<.001	<.001	<.001

n.s., not statistically significant.

displayed a follicular or partially follicular growth pattern. Twenty-four tumors were classified as FL grades 1 or 2, and 23 FLs as grade 3A, the latter without a significant (<25%) diffuse component. Of 27 FLs consisting of centroblasts exclusively, 5 displayed an exclusively follicular growth pattern (FL3B), whereas in 22 cases an additional DLBL component was present. In these FL3B+DLBL a follicular growth pattern was observed in at least 25% of the area infiltrated and confirmed by the presence of follicular dendritic cell meshworks. Figure 1, a to d, illustrates examples of FL grade 3A and 3B. One hundred fourteen (61%) lymphomas were classified as DLBL with a purely diffuse or sometimes vaguely nodular infiltrate of large B cells. Twenty-seven (14%) DLBLs were of primary extranodal origin arising at different sites (18 stomach, 5 thyroid gland, and 1 case each from the lung, small bowel, testis, and breast). Of the 87 nodal DLBLs, 77 arose *de novo*, whereas 10 additional nodal tumors had a history of a prior FL and were classified as transformed FL.

Immunohistochemistry

FLs grades 1, 2, and 3A proved to be quite homogeneous regarding the immunohistochemical expression profile of CD10, BCL-2, and BCL-6, but there was a certain decline in the reactivity for these parameters in FL3A (Table 1). P53 overexpression was found in few FL3A (3 of 23, 13%), whereas monotypic cytoplasmatic immunoglobulin expression (Clg+) was more frequent in FL3A (9 of 22, 41%) than in FL1/2 (3 of 24, 13%) ($P < 0.05$).

Compared with FL3A, FLs consisting of large cells exclusively (FL3B with or without a diffuse large B-cell component) harbored fewer cases with reactivity for CD10 (10 of 27, 37%) and BCL-6 ≥ 70% (12 of 27, 44%), but showed a high number of Clg+ cases (19 of 27, 70%) ($P < 0.05$). Purely follicular FL3B were consistently p53-negative, but Clg-positive in all cases. Transformed FLs were CD10-positive in only 50% of cases and showed the highest number of cases with p53 overexpression (40%).

Cytogenetic and Molecular Genetic Data

The translocation t(14;18)(q32;q21) was detected by classical cytogenetics or FISH in 21 of 24 (88%) FL1/2, but in fewer FL3A (64%) (Figure 1e). Altogether, 8 of 22 (36%) FL grade 3A were t(14;18)-negative (Table 2). If only specimens from untreated patients were considered, the t(14;18) was encountered in 14 of 17 (82%) FL1/2 and in 8 of 13 (62%) FL3A. In contrast, 3q27 rearrangements were not detected in a single case of FL 1/2, whereas four FL3A (17%) harbored the translocation. All five FL3B with a purely follicular growth pattern were negative for both t(14;18) and 3q27 alterations (Table 3). FL3B with a DLBL component (FL3B+DLBL) were t(3q27)-positive in more than 50% of cases (12 of 22, 55%; and 10 of 18 specimens from untreated patients, 56%) (Figure 1f). Remarkably, the frequency of this chromosomal alteration in FL3B with a DLBL component was significantly higher than that ascertained in purely diffuse DLBL of nodal (19 of 77, 25%) or extranodal (2 of 27, 7%) origin. Of importance, the differences regarding the occurrence of 3q27 alterations between FL3A, FL3B+DLBL, and diffuse LBL remained also statistically significant, when only untreated specimens were considered (Table 2).

Interestingly, among 19 nodal *BCL6* rearranged diffuse large B-cell lymphomas, we identified 6 cases (32%), in which focal remnants of CD21- and/or CD23-positive follicular dendritic cell meshworks were still present. The t(14;18) was found in 8 of 10 transformed FLs and in 9 of 77 (12%) *de novo* nodal DLBLs. It was not detected in a single case of primary extranodal DLBL (0 of 27). Three FL3A and each one case of FL transformed and nodal DLBL showed both the t(14;18) and a 3q27 rearrangement.

All 11 FL 1-3A negative for the t(14;18) by classical banding analysis were also FISH-negative excluding cryptic *BCL-2* rearrangements in FLs with preserved maturation to centrocytes. With respect to the detection of 3q27 alterations, however, FISH turned out to be dis-

Table 2. Cytogenetic Findings in 188 B-NHLs

Diagnosis	3q27 alteration		t(14;18)+	del TP53	del P16 ^{INK4a}	del D13S25	del D13S25/ TP53/P16 ^{INK4a}
	All pts.	Untreated pts.					
FL1/2 (n = 24)	0/24 (0%)	0/17 (0%)	21/24 (88%)	1/24 (4%)	0/24 (0%)	0/24 (0%)	1/24 (4%)
FL3A (n = 23)	4/23 (17%)	2/13 (15%)	14/22 (64%)	5/22 (22%)	3/22 (14%)	1/22 (5%)	8/22 (36%)
FL3B (n = 5)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
FL3B + DLBL (n = 22)	12/22 (55%)	10/18 (56%)	1/22 (5%)	6/22 (27%)	1/22 (5%)	2/22 (9%)	9/22 (41%)
FL transformed (n = 10)	2/10 (20%)	1/5 (20%)	8/10 (80%)	4/10 (40%)	2/10 (20%)	2/10 (20%)	5/10 (50%)
DLBL nodal (n = 77)	19/77 (25%)	16/67 (24%)	9/77 (12%)	20/77 (26%)	8/77 (10%)	12/77 (16%)	27/77 (35%)
DLBL extranodal (n = 27)	2/27 (7%)	2/25 (8%)	0/27 (0%)	6/27 (22%)	2/27 (7%)	2/27 (7%)	8/27 (30%)
p FL1/2 versus FL3A	<.05	n.s.	n.s.	n.s.	n.s.	n.s.	<.001
p FL3A versus FL3B + DLBL	<.001	<.05	<.001	n.s.	n.s.	n.s.	n.s.
p FL1-3A versus FL3B ± DLBL	<.001	<.001	<.001	n.s.	n.s.	n.s.	n.s.
p FL3B ± DLBL versus DLBL	<.001	<.05	n.s.	n.s.	n.s.	n.s.	n.s.

n.s., not statistically significant; pts.: patients.

tinctly more sensitive than classical cytogenetic and Southern blotting analyses. Whereas all specimens with 3q27 alterations in banding analyses also harbored *BCL6* rearrangements using FISH, additional *BCL6* split signals were also detected in 12 cytogenetically negative cases. *BCL6* rearrangements could be demonstrated in 23% (12 of 52) of cases analyzed by Southern blotting. These findings could be confirmed by FISH analyses in only eight of these tumors (67%). Additional *BCL6* alterations were detected by FISH in six Southern blot-negative cases. A comparison of the results in the individual cases is given, along with the diagnoses, in Table 4.

Correlations Between Cytogenetics and Immunohistochemistry for BCL-6

There was no positive correlation between a high expression of the BCL-6 protein and the presence of *BCL6* alterations neither in cases with a t(3;14)(q27;q32) nor in cases with other 3q27 alterations. The expression of BCL-6 was high ($\geq 70\%$ of cells) in the vast majority of FL1-3A (43 of 46; 93%) with and without the t(14;18). In contrast, the number of BCL-6 protein-positive cells in 3q27 nonrearranged and rearranged FL3B+DLBL and

DLBL cases was generally lower than in FL1-3A with or without 3q27 rearrangements (Table 5).

Alterations of p16^{INK4a}, TP53, and D13S25 Loci

Deletions of p16^{INK4a}, TP53, and/or D13S25 were rare events in FL1/2 (1 of 24, 4%) in contrast to 8 of 22 (36%) in FL3A ($P < 0.001$). FL3B, interestingly, failed to show any of these deletions, while they were common genetic events in FL3B+DLBL (41%, 9 of 22), nodal DLBLs (35%, 27 of 77), extranodal DLBLs (30%, 8 of 27), and transformed FLs (50%, 5 of 10). Deletion of TP53 was the most common genetic alteration and occurred especially in transformed FLs (40%) (Table 2).

Stratification of Lymphomas According to Primary Genetic Alterations

To elucidate the biological implications of the secondary genetic alterations in lymphoma progression, the tumors were stratified according to grade and the presence of primary chromosomal aberrations (Table 6). t(14;18)-positive FL1-3A were uniformly CD10-positive, with the

Table 3. Cytogenetic and FISH Data from Five Purely Follicular Cases FL3B

Case no.	Classical banding analyses	t(3q27) FISH	t(14;18) FISH
1	45-48,XY,dup(1)(q21q32)[2],dup(3)(p21p26)[9],dup(3)(p21p26)[4],+5[3],add(6)(q27)?t(6;8)(q27;q13)[9],i(12)(q10)[9]	Neg.	Neg.
2	42-54,XX,t(6;7)(p21;q22)[7],+7[7],+der(7)t(6;7)(p21;q22)[7],t(8;14)(q24;q32)[7],+11[5],+11[2],+12[4],+der(14)t(8;14)(q24;q32)[7],der(21)t(1;21)(q12;p13) × 2[7],+21[7]	Neg.	Neg.
3	44-46,XX,der(18)t(1;18)(q21;q23)[8] 45-46,X,der(X)t(X;1)(q28;q12)[8]	Neg.	Neg.
4	42-44,XY,i(1)(q10),t(3;6)(p21;p22),add(14)(q32),-6,t(7;9)(q21;q32),der(18)t(?1;18)(q32;q21),+mar[6]	Neg.	Neg.
5	Not done	Neg.	Neg.

Table 4. Cases Analyzed for *BCL-6* Rearrangements by Southern Blotting in Comparison with FISH and Cytogenetic Results

Case no.	Diagnosis	Southern blotting (<i>Xba</i> I/ <i>Bam</i> HI) (12 positive/52)	FISH (14 positive/52)	Cytogenetics (8 positive/44)
1	FL3B	G/G	Negative	No alteration
2	FL3B + DLBL	G/G	Negative	No alteration
3–19	DLBL nodal	G/G	Negative	No alteration (<i>n</i> = 14) n.d. (<i>n</i> = 3)
20–34	DLBL extranodal	G/G	Negative	No alteration (<i>n</i> = 12) n.d. (<i>n</i> = 3)
35	DLBL nodal	G/R	Positive	t(3;14)(q27;q32)
36	DLBL nodal	G/R	Positive	del(3q27)
37	FL3B + DLBL	G/R	Positive	t(3;14)(q27;q32)
38	FL2	G/R	Negative	No alteration
39	FL3B + DLBL	G/R	Positive	No alteration
40	FL3B + DLBL	R/G	Negative	dup(3)(q21;q27)
41	DLBL transformed	G/R	Negative	n.d.
42	DLBL nodal	G/G	Positive	t(3;16)(q27;p13)
43	DLBL nodal	G/R	Positive	No alteration
44	DLBL nodal	R/R	Positive	No alteration
45	DLBL nodal	G/G	Positive	t(3;12)(q27;p11)
46	DLBL nodal	G/R	Positive	No alteration
47	DLBL nodal	G/G	Positive	n.d.
48	DLBL nodal	G/G	Positive	t(3;14)(q27;q32)
49	DLBL nodal	G/G	Positive	t(3;14)(q27;q32)
50	DLBL extranodal	G/R	Positive	No alteration
51	DLBL extranodal	G/*	Positive	No alteration
52	DLBL extranodal	G/R	Negative	No alteration

n.d., not done; G, germline; R, rearranged.
 *Not available.

exception of one case BCL-2-positive and generally expressed BCL-6 in $\geq 70\%$ of cells. Three of eight (38%) t(14;18)-negative FL3A were p53-positive in contrast to 0 of 35 FL with the translocation ($P < 0.001$). Deletions of *TP53*, *p16*^{INK4a}, and/or *D13S25* altogether were infrequent in t(14;18)-positive FL1–3A (14%), but occurred more often in FL3A than in FL1/2 (29% versus 5%, $P < 0.05$). Interestingly, four of eight (50%) of t(14;18)-negative FL3A displayed *TP53* deletions. Of note, transformed FL did not harbor deletions of the above-mentioned loci in higher frequencies than FL3A.

The 10 nodal t(14;18)+ *de novo* large B-NHLs (with either a purely diffuse or a follicular and diffuse growth pattern) harbored deletions in one or more of the three chromosomal loci in 70% of cases, especially deletions of

TP53 in 6 of 10 tumors. In comparison with t(3;14)+ and t(14;18)–/t(3;14)– aggressive lymphomas, they showed significantly less cases with a plasmacytoid phenotype (C1g+), but were more often CD10- and BCL-6-positive (70%). Deletions of *TP53*, *p16*^{INK4a}, and/or *D13S25* occurred more often in t(14;18)+ *de novo* LBL than in t(3;14)+ and t(14;18)–/t(3;14)– tumors, especially deletions of *TP53* ($P < 0.05$). Remarkably, t(3;14)+ and t(14;18)–/t(3;14)– aggressive lymphomas did not differ significantly in any of the parameters detailed in Table 6. In particular, there was no difference in CD10 or BCL-6 expression between *BCL6* rearranged and nonrearranged FL3B+DLBL or DLBL, thus indicating that *BCL6* rearrangements are not significantly associated with a germinal center B-cell phenotype.

Table 5. Correlation of *BCL6* Rearrangements and BCL-6 Protein Expression

Diagnosis	BCL-6 expression $\geq 70\%$	
	3q27/t(3;14) positive	3q27/t(3;14) negative
FL1-3A <i>n</i> = 46	4/4 (100%)	39/42 (93%)
FL3B <i>n</i> = 5	0%	3/5 (60%)
FL3B + DLBL <i>n</i> = 22	4/12 (33%)	5/10 (50%)
DLBL nodal <i>n</i> = 76	7/19 (37%)	25/57 (44%)
DLBL extranodal <i>n</i> = 26	1/2 (50%)	8/24 (33%)

Discussion

FL grade 3, as defined in the World Health Organization classification,³ has recently turned out to be a heterogeneous entity with respect to morphology and genetics. We have shown that FL3A is a t(14;18)-positive neoplasm, whereas FL3B (without centrocytes) tend to harbor few rearrangements involving *BCL2*, but are frequently t(3q27)-positive.⁴ A recent report by Bosga-Bouwer and associates¹⁷ corroborated this latter finding. This biological heterogeneity of FL3 is possibly reflected in the long-standing controversy regarding the biological implication of FL grade 3 with clinical heterogeneity and different recommendations for treatment.^{18–21}

Table 6. Immunohistochemical and Genetic Findings in 188 B-NHLs Stratified According to Diagnosis and Genetic Alteration

	FL1/2 t(14;18)+ (n = 21)	FL3A t(14;18)+ (n = 14)	FL3A t(14;18)- (n = 8)	FL1-3A t(14;18)+ (n = 35)	FL1-3A t(14;18)- (n = 11)	FL transformed t(14;18)+ (n = 8)	t(14;18)+ de novo LBL (n = 10)	t(3;14)+ de novo LBL (n = 33)	t(3;14)-/ t(14;18)- de novo LBL (n = 88)
CD10	21/21 (100%)	14/14 (100%)	7/8 (88%)	35/35 (100%)	10/11 (91%)	5/8 (63%)	7/10 (70%)	9/33 (27%)	27/88 (31%)
BCL6 ≥ 70%	21/21 (100%)	12/13 (92%)	6/8 (75%)	33/34 (97%)	9/11 (82%)	6/7 (86%)	7/10 (70%)	12/33 (36%)	34/86 (40%)
Bcl-2	21/21 (100%)	13/14 (93%)	6/8 (75%)	34/35 (97%)	9/11 (82%)	8/8 (100%)	10/10 (100%)	26/33 (79%)	59/87 (68%)
P53 >20%	0/21 (0%)	0/14 (0%)	3/8 (38%)	0/35 (0%)	3/11 (27%)	4/7 (57%)	2/10 (20%)	9/33 (27%)	21/88 (24%)
del TP53	1/21 (5%)	2/14 (14%)	3/8 (38%)	3/35 (9%)	3/11 (27%)	3/8 (38%)	6/10 (60%)	6/33 (18%)	20/88 (23%)
del TP53/ p16 ^{INK4a} /D13	1/21 (5%)	4/14 (29%)	4/8 (50%)	5/35 (14%)	4/11 (36%)	4/8 (50%)	7/10 (70%)	9/33 (27%)	29/88 (33%)

The high frequency of *BCL2* rearrangements in FL3A is well in line with the identical cytological and immunological (CD10+, BCL-6+, BCL-2+) composition of these lymphomas as compared to FL grades 1 and 2. In contrast, the high frequency of *BCL6* aberrations in FL3B reported from different groups merits attention, because this alteration has been classically associated with DLBL (in ~30 to 40% of cases²²⁻²⁴). In an extensive interphase cytogenetic study of 188 well-characterized lymphomas including 74 FLs of different grades and 114 nodal and extranodal DLBLs, the t(14;18) was demonstrated by interphase FISH or cytogenetics in 21 of 24 (88%) FL1/2 and 14 of 22 (64%) FL3A. In contrast, all five FL3B with a purely follicular growth pattern were negative for both *BCL2* rearrangements and translocations affecting 3q27, and only a single case of FL3B+DLBL harbored the t(14;18). Strikingly, 12 of 22 FL3B with a DLBL component (55%) displayed breaks at 3q27. These cytogenetic differences were also mirrored in a differing immunophenotype, with FL3B harboring significantly fewer CD10-positive cases (37%) and also fewer cases with a high nuclear expression of BCL-6 (44%; Table 1). The translocation t(14;18)(q32;q21), therefore, which is regarded to be the genetic hallmark of FL,^{25,26} is typically associated with FL grades 1/2 and FL grade 3A. In this respect, it is noteworthy that additional FISH analysis, that was reported to be superior to classical cytogenetic banding or polymerase chain reaction analyses^{27,28} for the detection of *BCL2* rearrangements, was performed in all cytogenetically t(14;18)-negative FL1-3A in our study and failed to reveal cryptic *BCL2* rearrangements occasionally encountered in FL.²⁹ Irrespective of the presence of the t(14;18), all FL1/2 and 87% of FL3A were BCL-2 protein-positive. Despite the absence of *BCL2* rearrangements 75% of t(14;18)-negative FL3A expressed the BCL-2 protein, in agreement with previous reports.^{17,30-32} Interestingly, the only parameter distinguishing t(14;18)-positive and -negative FL with a centrocyte component in our series was the presence of p53 overexpression detected exclusively in t(14;18)-negative FL3A, pointing to a possible role of TP53 inactivation in these tumors (Table 6).

The finding that rearrangements affecting *BCL6* are especially frequent in NHLs with a follicular and diffuse

component and consisting of centroblasts exclusively (12 of 22, 55%) points to a substantial biological difference of FL3B+DLBL in comparison with FL1/2 and especially FL3A. Because all FL3B without a significant diffuse large B-cell component in our series were both t(14;18)- and t(3q27)-negative, these particular and rare FL variants may also be genetically different from FL3A and FL3B+DLBL, but too few data are yet available to allow for a definite conclusion. In particular, it is difficult to compare these results to the recent reports of two other groups, because neither Horsman and colleagues³¹ nor Bosga-Bouwer and associates¹⁷ give details about a DLBL component in their 3q27 rearranged 6 and 10 FL3B cases. *BCL6* gene rearrangements are frequent findings in cases classified as diffuse large B-cell lymphomas. Cytogenetic studies revealed chromosomal alterations affecting band 3q27 in up to 12 to 18% of DLBLs,³³⁻³⁶ whereas the overall incidence of 3q27 gene rearrangements in DLBL has been estimated to be 34% by Southern blot analyses.²²⁻²⁴ *BCL-6* rearrangements were also demonstrated in up to 14% in FL^{37,38} and infrequently in other lymphoma subtypes.³⁹⁻⁴¹

Because of the high frequency of *BCL6* rearrangements in FL3B+DLBL and the striking differences between the results of cytogenetic and Southern blot analyses reported in the literature, we also analyzed 77 nodal and 27 extranodal DLBLs for *BCL6* rearrangements using FISH, a method that has been regarded as the gold standard in identifying the t(3;14) and variants.^{5,6} Alterations of the *BCL6* gene locus were detected by FISH in 19 of 77 (25%) nodal DLBLs and in only 2 of 27 (7%) DLBLs with a documented extranodal primary origin resulting in an overall frequency of 21 of 104 (22%). The striking difference in the frequency of 3q27 rearrangements in nodal and extranodal lymphomas alone may explain the varying frequencies of *BCL6* alterations given in the literature, because the primary site of origin in DLBL is only rarely taken into account. Notwithstanding this important point, the frequency of *BCL6* rearrangements in FL3B+DLBL (55%) is significantly higher than that encountered in diffuse large B-cell lymphomas (22%) ($P < 0.001$), even if only nodal DLBLs are evaluated. Interestingly, we found remnants of follicular dendritic cell

meshworks in 6 (32%) of the 19 nodal DLBLs displaying a diffuse growth pattern on conventional stainings and displaying the t(3q27), allowing for the speculation that these diffuse large B-cell lymphomas also arose in the background of a follicular growth pattern. Rearrangements involving 3q27/*BCL6* constitute, therefore, the most frequent cytogenetic alteration in FL3B with a DLBL component and it is likely that a follicular growth component in predominantly diffuse LBLs may have been missed or disregarded in previous reports. A recent finding describing recurrent 3q27 alterations in nodular lymphocyte predominance Hodgkin lymphoma (NLPHL) underlines the possible significance of *BCL6* rearrangements in lymphomas with a follicular background. Wlodarska and associates⁴² detected the translocation by FISH in 48% of their 23 NLPHL cases investigated and interpreted this finding as consistent with a follicular origin of NLPHL in contrast to classical Hodgkin lymphoma.

It is currently unclear whether FL3B+DLBL represent lymphomas with a particular propensity to home into, or colonize, pre-existing reactive germinal centers, or if these tumors arise in germinal centers.⁴³ The significantly higher frequency of *BCL6* rearrangements in FL3B+DLBL in comparison to purely diffuse LBLs might be considered an argument in favor of the latter hypothesis. The t(3;14)(q27;q32) and variants lead to rearrangements of the *BCL6* proto-oncogene, encoding a POZ/zinc-finger transcriptional repressor.^{44–47} The BCL-6 protein is expressed by mature B cells in the germinal center, whereas it is negative in precursor B cells and postfollicular B cells. *BCL6* is essential for the development of the germinal center and for antigen-dependent immunological response.^{48–52} In the reactive germinal center, the function of *BCL6* as a transcriptional repressor is based on its interaction with Blimp-1 (B-lymphocyte-induced maturation protein 1), a transcriptional repressor that plays a key role in the differentiation of activated B cells to mature plasma cells.^{53,54} Blimp-1 acts as a transcriptional repressor of *cMYC* and also causes growth arrest and terminal plasmacytic differentiation or apoptotic death. Repression of Blimp-1, therefore, by deregulated expression of BCL-6 might be important in lymphomagenesis⁵⁴ permitting continued cell growth. This deregulated function of *BCL6*, at present, is thought to be induced by either chromosomal rearrangements or mutations of the gene.^{55–58} The results of the present investigation suggest that *BCL6* deregulation induced by chromosomal translocations leads to a particular type of aggressive B-NHL characterized by a partly preserved follicular architecture, a finding that is consistent with the importance of *BCL6* for germinal center formation and function. Therefore, these FL3B+DLBL frequently characterized by 3q27 alterations might be considered germinal center-related neoplasms. In the present series, however, proteins such as CD10 and/or BCL-6, markers of the germinal center B-cell-like group of aggressive large B-cell lymphomas^{59,60} were not expressed more often in FL3B or *BCL6* rearranged cases in comparison to DLBL or *BCL6* rearrangement-negative tumors. Similar to the recent findings of Jardin and colleagues,⁶¹ the number of BCL-6 protein-positive cells in 3q27 rearranged

and nonrearranged FL3B+DLBL and DLBL cases in our series was not significantly different and, in contrast, was even lower than in typical FL grades 1–3A negative for the translocation (Table 5). Therefore, lower BCL-6 expression in ambiguous cases can be considered a marker for FL3B.

Interestingly, we failed to identify profound differences in immunohistochemical features and the occurrence of secondary chromosomal alterations between aggressive B-cell lymphomas with or without 3q27 rearrangements. In contrast, large B-cell lymphomas arising *de novo* with a t(14;18) were different from both t(3;14)-positive and t(14;18)/t(3;14) double-negative LBL. These findings are in line with previous reports^{59,62} demonstrating that *BCL2* rearranged *de novo* LBLs constitute a particular subgroup of LBL with germinal center-like features as defined by gene expression profiling.

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