Follicular lymphoma grade 3B is a distinct neoplasm according to cytogenetic and immunohistochemical profiles

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

According to the current World Health Organization Classification of Lymphoid Tumours, follicular lymphoma is subclassified into three grades according to the number of centroblasts. Follicular lymphoma grade 3 can be further divided into types A and B. Almost all available genetic data on grade 3B follicular lymphomas have been generated from tumors with an additional diffuse large B-cell lymphoma component. The purely follicular type of follicular lymphoma grade 3B is a rare neoplasm.

Design and Methods

We performed a detailed immunohistochemical (CD10, IRF4/MUM1, BCL2, Ig light chains) and genetic (translocations of *BCL2*, *BCL6*, *MYC*, *IRF4*) characterization of the largest series of purely follicular cases of grade 3B follicular lymphoma available to date, comprising 23 tumor samples. We also included 25 typical grade 1 or 2 follicular lymphomas, 9 follicular lymphomas with large centrocytes and/or high proliferation indices (FL/LCC), 12 cases of follicular lymphoma grade 3A, 16 cases of diffuse large B-cell lymphoma/follicular lymphoma grade 3B and 15 follicular lymphomas in which a straightforward distinction between grades 3A and 3B was not possible.

Results

Translocations affecting *BCL2* and *BCL6* genes are rare in grade 3B follicular lymphomas (2/23, 9% and 4/23, 17%) when compared with grade 1 or 2 follicular lymphomas (22/25, 88%, $P<0.001$ and 0/25, $P<0.05$), FL/LCC (7/9, 78%, $P<0.001$ and 2/9, 22%), grade 3A follicular lymphomas (7/12, 58%, $P<0.01$ and 2/12, 17%), unclassified grade 3 follicular lymphomas (6/15, 40% and 2/15, 13%) and diffuse large B-cell lymphoma/follicular lymphoma grade 3B (2/16, 13% and 8/16, 50%, $P<0.05$). *MYC* translocations were detected occasionally in FL/LCC, follicular lymphoma grade 3B, and diffuse large B-cell lymphoma/follicular lymphoma grade 3B (13%-22%), but not in grade 1, 2 or 3A follicular lymphomas ($P<0.05$ when compared with follicular lymphoma grade 3B). Both follicular lymphoma grade 3B and diffuse large B-cell lymphoma/follicular lymphoma grade 3B were enriched in samples with a $CD10^-IRF4/MUM1^+$ immunophenotype (8/19, 42% and 7/16, 44%), with the vast majority of them lacking BCL2 translocations. In contrast, 42/46 grade 1 or 2 follicular lymphomas, FL/LCC and grade 3A follicular lymphomas were $CD10^+$ (91%) while 0/46 expressed IRF4/MUM1. None of the tumor samples tested with increased IRF4/MUM1-expression harbored a translocation of the *IRF4* gene locus.

Conclusions

Our results show that grade 3B follicular lymphomas form a distinct category of follicular lymphomas with infrequent *BCL2* and *BCL6* translocations, while grades 1, 2 and 3A follicular lymphomas and FL/LCC display homogeneous features with frequent *BCL2* translocations and a $CD10^+IRF4/MUM1^-$ immunophenotype.

Key words: follicular lymphoma, grade 3B, FISH, immunohistochemistry, genetics.

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Introduction

Follicular lymphomas (FL) are the second most frequent entity of malignant lymphoma and have a broad range of morphologies, immunophenotypes, and cytogenetic constitutions. In the vast majority of cases, FL manifests as a systemic disease involving lymph nodes. By definition, the tumor shows an at least partly follicular growth pattern. Most cases of FL are composed mainly of centrocytes, with a few interspersed centroblasts [FL grades 1 and 2 according to the World Health Organization (WHO) classification].[1] Roughly 85% of these tumors are associated with the t(14;18)(q21;q32) chromosome translocation that constitutively deregulates and over-expresses the *BCL2* proto-oncogene. However, rarer subtypes of FL, often arising primarily in extranodal sites, in the pediatric population, or with varying architectural or cytological features, frequently display distinct clinical and/or cytogenetic features[1-4] in comparison with the classic nodal FL grade 1 (FL1) and 2 (FL2). Of these subtypes, FL grade 3 (FL3) have gained considerable interest because of their unresolved status as indolent or aggressive neoplasms, and their varying immunophenotypic, cytogenetic and possibly clinical features.[5-13] Currently, the WHO classification defines two types of FL3, one being composed of centroblasts and centrocytes (FL3A), the other harboring exclusively centroblasts (FL3B).[1] While FL3A more closely resembles FL1 and FL2, FL3B was found to display more variable immunophenotypic and genetic features.[10,11] We and others[5,6,10,11] have reported that patients with FL3B often do not have the t(14;18)/*BCL2* rearrangement and that the large majority of cases of this subtype of lymphoma have a diffuse large B-cell lymphoma (DLBCL) component (the former *centroblastic lymphoma, follicular and diffuse*, of the Kiel classification[14]). In the third edition of the WHO classification, FL3 was defined as a tumor with an at least partly follicular growth pattern harboring more than 150 centroblasts per ten high power fields. This definition was adopted in most reports describing the cytogenetic constitution of FL3B.[5,6,8-11] The updated WHO classification of 2008, however, explicitly states that the presence of a diffuse component in FL3 warrants a separate diagnosis of DLBCL. The vast majority of FL3B described in the literature would, therefore, fall into this category nowadays. The fact that cases of DLBCL associated with a FL3B component do frequently harbor translocations involving *BCL6*, one of the hallmark translocations of DLBCL, fits well with this concept.[5,6,10,11] The cytogenetic constitution of *purely* follicular FL3B, on the other hand, is largely unknown. In this study we, therefore, set out to define the largest series available to date of FL3B with immunophenotypic and cytogenetic data, also paying particular attention to their sometimes difficult differentiation from FL3A and related variants.

Design and Methods

Lymphoma specimens

Twenty-three patients with purely follicular FL3B, referred to the Department of Pathology at the University of Würzburg, Germany, the Department of Clinical Pathology, Robert-Bosch Krankenhaus, Stuttgart, Germany, the Department of Pathology, Caritas-Krankenhaus Bad Mergentheim, Germany, and the Department of Pathology, Hospital General de México, OD and Medical School, University of Mexico (UNAM) between 1992 and 2007, were classified according to the criteria of the WHO classification of tumors of hematopoietic and lymphoid tissues.[1] The study was approved by the local ethics committee, in accordance with the Declaration of Helsinki. The diagnosis of FL3B required an exclusively follicular growth pattern, verified by the presence of $CD21^+$ or $CD23^+$ follicular dendritic cell meshworks, and growth of centroblasts in solid sheets, without residual centrocytes.[1] We also studied 25 cases of FL1 or FL2 (FL1/2), 12 cases of FL3A and 16 cases of DLBCL with an additional FL3B component (DLBCL/FL3B) with regard to their delineation from purely follicular lymphoma FL3B. In addition, nine cases of FL1/2 with large centrocytes and/or high proliferation indices (FL/LCC) were included in the series because of their sometimes blastoid appearance and hence, overlap with FL variants with a higher blast content (Figure 1D).[15] Of those, four were classified as FL with large centrocytes (mean proliferation index, 32%; range, 20%-80%), and five as FL with high proliferation index (mean proliferation index, 44%; range, 30%-80%). In 15 (exclusively follicular) FL3, a straightforward and reproducible distinction between grades 3A and 3B was not possible. These cases were termed FL3 unclassified (FL3U; Figure 1C). Only primary diagnostic lymphoid biopsies from untreated patients were included in the study. *Online Supplementary Table S1* gives an overview of all cases included in the study. The median age of patients was 60 years (range, 9 to 86 years). Only one pediatric patient (9 years old) with a diagnosis of FL3 was identified in the present series. Because we did not, for reasons of principal, exclude pediatric patients from the series, we chose to keep this case in the study cohort. All cases were classified on the basis of routine hematoxylin and eosin, Giemsa and periodic acid Schiff staining and CD21 and/or CD23 immunostaining (see below).

Immunohistochemistry

Paraffin sections were immunostained for diagnostic purposes with antibodies directed against CD20 (clone L26, dilution 1:1000, DAKO, Glostrup, Denmark), CD5 (clone 4C7, dilution 1:40, Novocastra, Newcastle on Tyne, UK), Ki67 (MM1, dilution 1:30, Novocastra), BCL2 (clone 124, dilution 1:150, DAKO), and BCL6 (clone PG-B6p, dilution 1:25, DAKO). The presence of a follicular dendritic cell meshwork was determined by immunostaining for either CD21 (clone 1F8, dilution 1:200, DAKO) or CD23 (clone 1B12, dilution 1:80, Novocastra). Immunohistochemical studies with monoclonal antibodies directed against CD10 (NCL-CD10-270, dilution 1:5, Novocastra) and IRF4/MUM1 (Mum1P, 1:100, DAKO) were performed as previously described.[2,16] Plasmacytoid/secretory differentiation was made when unequivocal monotypic cytoplasmic reactivity for either κ- or λ-immunoglobulin light chains (dilutions 1:40.000 and 1:20.000, respectively, DAKO) (cIg+) was observed.[16] Staining for CD20, CD5, CD10, and BCL2 was scored as positive or negative, while staining for Ki67, IRF4/MUM1, and BCL6 was scored in a semi-quantitative manner indicating the percentage of positive tumor cells in increments of 10%. IRF4/MUM1 staining was considered positive if at least 30% of nuclei were stained. All immunohistochemical reactions were performed on paraffin sections using the DAKO Envision system after antigen retrieval.

Fluorescence in situ hybridization on interphase nuclei

Fluorescence *in situ* hybridization on interphase nuclei (interphase-FISH) was performed on 4 μm thick tissue sections freshly cut from the respective paraffin blocks. Hybridization and immunodetection were carried out as previously described.[17] Sections were hybridized with Vysis LSI® *BCL2*-, Vysis LSI®

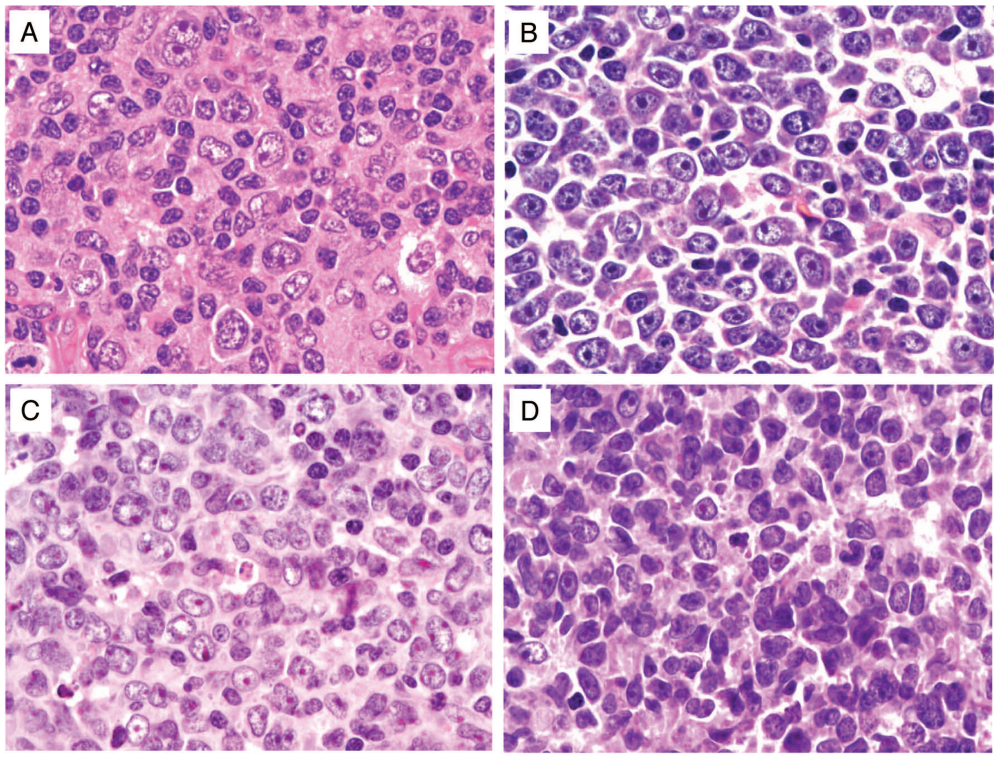

Figure 1. Examples of cytological details in FL3A (A), FL3B (B), FL3U (C) and FL/LCC (D). In FL3A, the centroblasts and centrocytes differ markedly in size and overall appearance (A). In FL3B, the blastic cells are homogeneous in size and shape and, in this case, many display immunoblast-like features (B). In FL3U, next to easily discernible blasts, some medium-sized cells are present that display characteristics of both centroblasts and centrocytes (C). FL/LCC harbors many cells with the overall appearance of centrocytes, but that are large and have open nuclear chromatin, giving them blastoid features (D).

BCL6 MBR (Minor Breakpoint Region), and Vysis LSI® *MYC*-dual color break-apart rearrangement probes (Vysis/Abbott Molecular Diagnostics, Wiesbaden-Delkenheim, Germany). Moreover, a recently developed *IRF4* break-apart probe was applied.[18] Tissue sections from reactive lymph node specimens were used to determine the cut-off level for each probe. The mean plus three times the standard deviation of the normal range was set as the reference range. At least 100 intact nuclei per case were evaluated on a fluorescence microscope (Leica Microsystems, Bensheim, Germany or Carl Zeiss, Jena, Germany). Illustrations were documented using the ISIS imaging system (MetaSystems, Altlussheim, Germany). The signal distribution was evaluated by two independent observers. The cut-off levels were 13%, 12%, 15% and 10% for the *BCL2*-, *BCL6*-, *MYC*- and *IRF4*-break-apart probes, respectively.

Statistical analysis

Statistical comparisons were done in GraphPad Prism (V3.0, GraphPad Prism Software Inc., San Diego, CA, USA). Different groups were compared by Student's t-test (in GraphPad Prism) and Fisher's exact test. *P* values less than 0.05 were considered statistically significant.

Results

Morphology and clinical data

The present series consisted of 100 cases: 25 FL1/2, 9 FL/LCC, 12 FL3A, 23 FL3B, 15 FL3U, and 16 DLBCL/FL3B. In most cases, two expert hematopathologists (GO and AR) easily reached consensus on the classification of the lymphoma. However, in 15 FL3, discussion of the cases without knowledge of immunophenotypic and genetic data revealed arguments in favor of both FL3A and FL3B. These cases had either been differently classified by the two main observers, or they were not consistently classified during different rounds of review. During multihead microscope discussions it turned out that the classification of cases as FL3A was straightforward if centroblasts and centrocytes differed markedly in size, and as FL3B, if the (large) centroblasts showed a homogeneous cytology (Figure 1A,B). Some cases of FL3 were, however, difficult to classify because of the smaller size of the blasts and the presence of a smaller cell population that showed intermediate features not allowing for their unequivocal characterization as "small blasts" or "large centrocytes" (Figure 1C). We chose, however, not to reach a consensus diagnosis, but to create a category of FL3U reflecting the obvious diagnostic uncertainty in some cases. Because of the retrospective nature of the study, no data on initial presentation or follow-up could be obtained. Data on the age and sex of the patients are given in *Online Supplementary Table S1*. No significant differences were noted between FL subgroups.

Genetic features

All cases were hybridized with break-apart probes flanking the breakpoints in the *BCL2*, *BCL6* and *MYC* genes. In addition, we investigated 28 cases with a newly generated *IRF4* break-apart probe.[18] *BCL2* breaks were the predominant genetic feature in FL1/2 (22/25, 88%), FL/LCC (7/9, 78%) and FL3A (7/12, 58%), and were also observed in a significant proportion of FL3U (6/15, 40%). They were, however, only infrequently detected in FL3B (2/23, 9%) and DLBCL/FL3B (2/16, 13%). Thus, FL3B can be clearly distinguished from FL1/2 and FL/LCC ($P<0.001$) and FL3A ($P<0.01$) on the basis of the distribution of *BCL2* gene rearrangements, while DLBCL/FL3B and FL3B do not differ significantly with regards to *BCL2* status (Table 1, Figure 2A).

Table 1. Frequency of breaks in the *BCL2*, *BCL6* and *MYC* loci, and staining patterns of BCL2, CD10, IRF4/MUM1 and monotypic cytoplasmic Ig light chains (cIg) in FL1/2, FL/LCC, FL3A, FL3B, FL3U and DLBCL with a FL3B component (DLBCL/FL3B).

Subgroup	*BCL2* Break	*BCL6* Break	*MYC* Break	BCL2⁺	CD10⁺	IRF4/MUM1⁺	cIg⁺
FL1/2	88% 22/25	0 0/25	0 0/25	96% 24/25	100% 25/25	0 0/25	13% 2/16
FL/LCC	78% 7/9	22% 2/9	22% 2/9	78% 7/9	78% 7/9	0 0/9	33% 3/9
FL3A	58% 7/12	17% 2/12	0 0/12	50% 6/12	83% 10/12	0 0/5	45% 5/11
FL3B	9% 2/23	17% 4/23	22% 5/23	45% 9/20	43% 9/21	42% 8/19	57% 12/21
FL3U	40% 6/15	13% 2/15	13% 2/15	53% 8/15	43% 6/14	14% 2/14	13% 2/15
DLBCL/FL3B	13% 2/16	50% 8/16	19% 3/16	44% 7/16	31% 5/16	63% 10/16	25% 4/16

In contrast, rearrangements of the *BCL6* gene locus were most frequently observed in DLBCL/FL3B (8/16, 50%), and were also detected in small numbers of FL/LCC (2/9, 22%), FL3B (4/23, 17%), FL3A (2/12, 17%) and FL3U (2/15, 13%). No *BCL6* breaks were observed within the group of FL1/2. Thus, the highest frequency of *BCL6* breaks was encountered in DLBCL/FL3B (8/16, 50%), with this frequency differing significantly from that in FL3B ($P<0.05$). There was no significant difference between FL/LCC, FL3A and FL3B with respect to their overall low frequency of *BCL6* rearrangement (Table 1, Figure 2B).

Signal constellations indicative of a break in the *MYC* gene locus were most frequently observed in FL3B (5/23, 22%), FL/LCC (2/9, 22%) and DLBCL/FL3B (3/16, 19%), and to a lesser extent in FL3U (2/15, 13%). No *MYC* gene alterations were detected in FL1/2 or FL3A. The incidence of *MYC* translocations was higher in FL3B than in either FL1/2 or FL3A ($P<0.05$, respectively). In general, *MYC* translocations were observed in FL/LCC, FL3B, FL3U and DLBCL/FL3B (Table 1, Figure 2B). As expected, FL3U showed a varying genetic constitution with regards to *BCL2*, *BCL6* and *MYC* status, reflecting the inconsistency in its morphological definition (Table 1, Figure 2). No breaks were observed for the *IRF4* break-apart probe in any of the 28 cases tested. The mean frequency of *MYC*, *BCL2* and *BCL6* was 57% (range, 35-82%), 66% (range, 40-84%) and 67% (range, 39-85%) of cells affected per sample, respectively.

Among 12/100 tumor samples (12%) harboring *MYC* translocations, the *MYC* rearrangement was the sole genetic event recognized in six samples, with four of these being in the FL3B subgroup. In five cases (42%), additional breaks in other genes tested were encountered, targeting BCL2 (*BCL2⁺/MYC⁺*) in one FL3U and in two FL/LCC. Dual translocations of *BCL6* and *MYC* (*BCL6⁺/MYC⁺*) were detected in two DLBCL/FL3B cases. Of the two FL/LCC double hit cases, one was classified as FL with large centrocytes, and one was a FL with a high proliferation index and blastoid features. Moreover, one FL3B was found to harbor a triple hit translocation of *BCL2*, *BCL6* and *MYC* simultaneously (*Online Supplementary Table S1*).

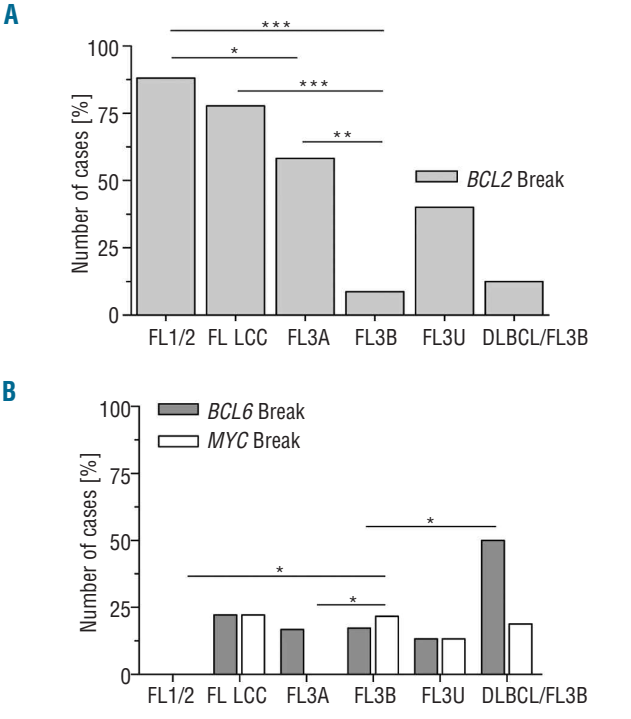

Figure 2. Distribution of genetic alterations in FL3B in comparison to FL subtypes FL1/2, FL/LCC, FL3A, FL3B, FL3U, and DLBCL with a FL3B component (DLBCL/FL3B). *P* values <0.05 were considered statistically significant (**P*<0.05; ***P*<0.01; ****P*<0.001). (A) Genetic alterations affecting the *BCL2* gene locus. The frequency of *BCL2* gene translocations was significantly higher in FL1/2, FL/LCC and FL3A when compared to FL3B (*P*<0.001, *P*<0.001 and *P*<0.01, respectively). FL1/2 and FL3A were different as regards *BCL2* translocation status (*P*<0.05). No significant differences were observed comparing FL3B with FL3U or DLBCL/FL3B. (B) Translocations of the *BCL6* and the *MYC* gene loci. A significantly higher number of *MYC* rearrangements was found in FL3B than in FL1/2 or FL3A (*P*<0.05). DLBCL/FL3B harbored a significantly higher proportion of *BCL6* rearrangements when compared to FL3B (*P*<0.05).

Immunophenotypic features

Immunohistochemically, FL1/2 was a homogeneous entity, with all cases being positive for CD10 and all but one expressing BCL2 (24/25, 96%). All FL1/2 were negative for IRF4/MUM1 (Table 1, Figure 3A,B). A comparable staining pattern was evident for FL/LCC (CD10: 7/9, 78%; BCL2: 7/9, 78%; IRF4/MUM1: 0/9) and FL3A (CD10: 10/12, 83%; IRF4/MUM1: 0/5), although only 50% (6/12) of FL3A were BCL2⁺ (Table 1, Figure 3A,B). In contrast, significantly higher proportions of FL3B cases expressed IRF4/MUM1 (8/19, 42%) and had reduced CD10 expression of the tumor cells (9/21, 43%) when compared with FL1/2, FL/LCC and FL3A ($P<0.001$, $P<0.05$ and $P<0.05$, respectively) (Table 1, Figure 3A). BCL2 expression was significantly more frequent in FL1/2 than in FL3B (9/20, 45%, $P<0.001$) (Table 1, Figure 3B). Of the 16 cases of DLBCL/FL3B, 10 (63%) expressed IRF4/MUM1, 5 (31%) were positive for CD10 and 7 (44%) were positive for BCL2 (Table 1, Figure 3A,B). Out of 32 CD10⁻ samples, 17 (53%) showed reactivity (≥30%) for IRF4/MUM1, 10 of them (10/32, 31%) with an exclusively follicular growth pattern (8/10 FL3B, 2/10 FL3U). In contrast, only 3/47 CD10⁺ tumors (6%) were IRF4/MUM1⁺, all of which were

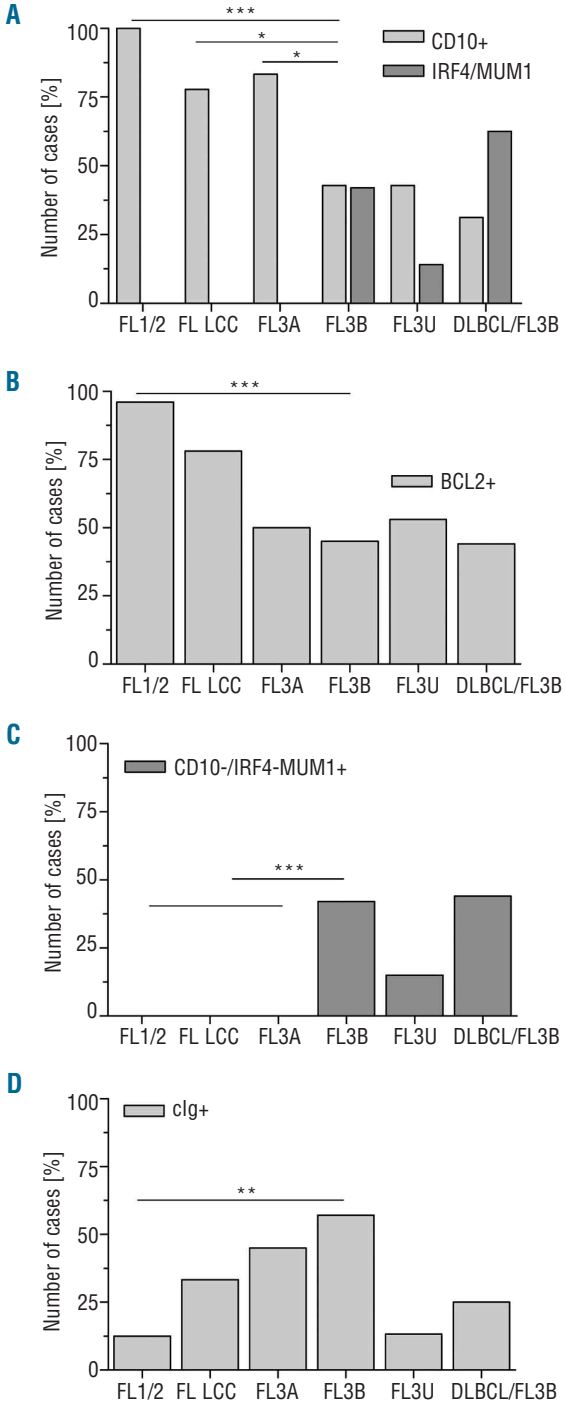

Figure 3. Results of immunohistochemical stainings for CD10, IRF4/MUM1, BCL2 and cytoplasmic Ig light chains (cIg$^+$) in FL3B compared to FL subtypes FL1/2, FL/LCC, FL3A, FL3B, FL3U and DLBCL with a FL3B component (DLBCL/FL3B). *P* values <0.05 were considered statistically significant (**P*<0.05; ***P*<0.01; ****P*<0.001). (A) CD10 and IRF4/MUM1 expression levels. The number of cases expressing CD10 was significantly higher in FL1/2, FL/LCC and FL3A than in FL3B (*P*<0.001, *P*<0.05 and *P*<0.05, respectively), while FL3B expressed IRF4/MUM1 unlike FL1/2, FL/LCC and FL3A (*P*<0.001, *P*<0.05 and *P*<0.05, respectively). (B) BCL2 staining patterns. FL1/2 harbored distinctly more cases expressing BCL2 than did FL3B (*P*<0.001). In contrast, BCL2 expression levels were comparable in FL/LCC, FL3A, FL3U and DLBCL/FL3B and FL3B. (C) Distribution of CD10$^-$IRF4/MUM1$^+$ immunophenotype. Significantly more FL3B showed a CD10$^-$IRF4/MUM1$^+$ staining pattern in contrast with FL1/2, FL/LCC and FL3A (*P*<0.001). (D) Immunohistochemical expression levels of cIg light chains. FL3B were frequently cIg$^+$, unlike FL1/2 (*P*<0.001).

DLBCL/FL3B (*Online Supplementary Table S1*). Cases with a CD10$^-$IRF4/MUM1$^+$ phenotype (n=17) had been classified as FL3B (8/19, 42%, *P*<0.001 when compared to FL1/2, FL/LCC and FL3A) or as DLBCL/FL3B (7/16, 44%), and to a lesser extent as FL3U (2/13, 15%). Neither enhanced IRF/MUM1 expression nor a CD10$^-$IRF4/MUM1$^+$ phenotype was observed in FL1/2, FL3A or FL/LCC (Tables 1 and 2, Figure 3C). A CD10$^+$IRF4/MUM1$^-$ phenotype, on the other hand, was a characteristic feature in FL1/2 (25/25, 100%), FL/LCC (7/9, 78%) and FL3A (4/5, 80%) (Table 2, Figure 3C). Interestingly, 16/17 CD10$^-$IRF4/MUM1$^+$ samples (94%) lacked the BCL2 translocation, the only exception being one FL3U (*Online Supplementary Table S1*). A substantial number of FL3B displayed plasmacytoid differentiation (cIg$^+$) (12/21, 57%), in contrast with FL1/2 (2/16, 13%) (*P*<0.01), and FL/LCC (3/9, 33%) (*P*=not significant). FL3A, however, were also frequently cIg$^+$ (5/11, 45%). No significant difference was observed between FL3B and DLBCL/FL3B (4/16, 25%) (Table 1, Figure 3D). Although plasmacytoid differentiation, together with decreased BCL2 expression and a CD10$^-$IRF4/MUM1$^+$ immunophenotype, was most frequently encountered in FL3B, no significant correlation of these phenotypic features was observed in our study cohort, with similar numbers of cases being cIg$^+$ and BCL2$^-$ (8/34, 23%), as well as BCL2$^+$ (20/52, 38%), or cIg$^+$ combined with either CD10$^+$IRF4/MUM1$^-$ (9/22, 41%) or CD10$^-$IRF4/MUM1$^+$ (13/22, 59%, *Online Supplementary Table S1*). Since IRF4/MUM1 over-expression might be caused by underlying genetic alterations of the *IRF4* gene locus,[18] 11 IRF4/MUM1$^+$ and 17 IRF4/MUM1$^-$ specimens were analyzed for breaks in the *IRF4* gene. However, none of the cases showed *IRF4* translocations (*Online Supplementary Table S1*). The mean proliferation index, as measured by Ki67 expression, was 29.6% in FL1/2 (range, 10-80%), 37.9% in FL/LCC (range, 10-80%), 68% in FL3A (range, 40-80%), 57.9% in FL3B (range, 20-90%), 42.6% in FL3U (range, 10-80%) and 79.4% in DLBCL/FL3B (range, 70-100%). The Ki67 index of FL3B was significantly different from that of FL1/2 (*P*<0.001) and DLBCL/FL3B (*P*<0.001), whereas no significant differences were observed between FL3B, FL/LCC, FL3A and FL3U (*data not shown*).

Discussion

The great majority of FL, as defined in the current WHO classification, form a homogeneous tumor entity, with the hallmarks of a (partly) follicular growth pattern, reactivity of the tumor B cells for CD10 and BCL6, and presence of a t(14;18)(q32;q21).[1] Certain disease variants, however, display varying immunohistochemical and cytogenetic features, which, next to FL occurring in pediatric populations or in extranodal localizations, have been especially noted in FL3B.[5,6,11] Almost all available data concerning these FL3B have, however, been generated from tumors with an additional DLBCL component,[5,6,8-11] and these

Table 2. Distribution of CD10+IRF4/MUM1- and CD10-IRF4/MUM1+ immunophenotypes in the FL subgroups.

Subgroup	CD10+MUM1/IRF4-	CD10-MUM1/IRF4+
FL1/2	100% 25/25	0 0/25
FL/LCC	78% 7/9	0 0/9
FL3A	80% 4/5	0 0/5
FL3B	42% 8/19	42% 8/19
FL3U	46% 6/13	15% 2/13
DLBCL/FL3B	13% 2/16	44% 7/16

cases would nowadays be diagnosed as a DLBCL with an associated follicular FL3B component (DLBCL/FL3B).[1] The case set of the present study is, therefore, unique in that it represents the largest number of *purely follicular* FL3B reported on to date with available genetic and immunophenotypic data (n=23). In addition, we compared our molecular cytogenetic and immunohistochemical data from this exceedingly rare FL subset with those of another less well-studied group of FL, tumors predominantly composed of large centrocytes and/or displaying higher Ki67 indices (>30%, FL/LCC).[15]

The main cytogenetic finding in our present case series, unrelated to our previously reported samples,[2,10,11] was that FL3B only infrequently harbored a t(14;18) in contrast to FL1/2, which predominantly included translocation-positive tumor samples, and FL3A, thus confirming our published data.[2,10,11,19] Furthermore, we confirmed that *BCL6* rearrangements are frequently encountered in DLBCL/FL3B, but are – again - a rare finding in FL3B.[10] These results, however, are not in agreement with the findings of Bosga-Bouwer *et al.*,[5,6] who reported *BCL2* and *BCL6* rearrangements in 37% and 33% of cases of FL3B, respectively. The higher frequency of *BCL6* rearrangements in their series can be easily explained by the fact that Bosga-Bouwer *et al.* mainly described cytogenetic findings in FL3B with a significant diffuse component, as stated in the respective papers.[5,6] These cases would nowadays be classified as DLBCL with a FL3B component[1] and are, therefore, well comparable to our DLBCL/FL3B samples. The frequent t(14;18)/*BCL2* rearrangements in their series (37%) were not, however, found in our previous studies[10,11] or in the present series. We believe that there are two possible explanations for this difference. First, the categorization of FL with large centrocytes (frequently with higher proliferation indices) is not entirely straightforward, since some hematopathologists may regard such cells as "large centrocytes", while others may consider them as "small blasts". However, most experts will regard these cases as part of the spectrum of FL1/2.[15] In fact, we found that FL/LCC do show typical FL1/2 features such as a CD10+IRF4/MUM1- immunophenotype, expression of BCL2 protein and frequent rearrangements of the *BCL2* gene. Second, six cases in the series reported by Bosga-Bouwer *et al.* in 2006,[6] which had been diagnosed as having FL1/2, were reclassified as having FL3B during the course of their disease, and four of these six were t(14;18)-positive. The reason for reclassification was not specified and, especially, it was not stated whether they had been reclassified because of transformation. The reclassification could, therefore, reflect diagnostic inconsistency or indicate a higher grade transformation of low-grade disease. For reasons of principal we had excluded transformed FL – which may well grow in follicular structures - from our series. Furthermore, in contrast to the other series, we created a category of FL3U among our cases. It is not clear why the problem of subclassifying some cases of FL3A was not addressed in previous reports of FL3.[13,20] Most probably, all the cases were assigned to one of the two groups, because an FL3U group was not defined and therefore, an unequivocal classification had to be made.

Concerning the analysis of *MYC* rearrangements and the distribution of "double hit" or "triple hit" cases in our series, it is interesting to note that *MYC* translocations were detected – albeit at low frequencies – in all categories of FL (13%-22%) with the exception of FL1/2 and FL3A. This finding agrees well with the data from other studies, in which *MYC* translocations were found in up to 13% of cases.[6,21-25] It is especially interesting to note that the incidence of *MYC*+ cases was the same in the FL/LCC subtype as in the FL3B and DLBCL/FL3B subtypes, making *MYC* a likely candidate as a progression factor also in "non-transformed" FL. Out of 12 cases of FL with *MYC* gene translocation, five cases presented with concurrent translocations of either *BCL2* or *BCL6*, while one case of FL3B showed a triple hit of *MYC*, *BCL2* and *BCL6*. These data are well in line with those of a recent study, analyzing *MYC*+ lymphomas in the Mitelman database.[26] It was concluded that about 50% of *MYC*-rearranged tumors are double-hit lymphomas, predominantly comprising aggressive B-cell lymphomas such as Burkitt's lymphoma and DLBCL, but to a lesser extent also including FL. While the great majority of double-hit lymphomas simultaneously harbored *BCL2* and *MYC* (*BCL2*+/*MYC*+) rearrangements, a dual translocation of *BCL6* and *MYC* (*BCL6*+/*MYC*+) was only rarely encountered,[26] as in the present series. Although it has been reported that translocation of *MYC*, as either a single- or double-hit event, is associated with highly aggressive and transformed variants of lymphoma,[26] rare *BCL2*+/*MYC*+ double-hit cases have been identified in FL described as having "blastoid" morphology.[23,27] There are, however, few data available concerning the clinical consequences of a *BCL2*+/*MYC*+ double-hit in non-aggressive FL.

The genetic differences between FL1/2, FL/LCC and FL3A on the one hand, and FL3B and DLBCL/FL3B on the other hand were also mirrored by their immunohistochemical status. Fairly high percentages of both FL3B and DLBCL/FL3B had a CD10-IRF4/MUM1+ immunophenotype (8/19, 42% and 7/16, 44%, respectively), whereas 42/46 FL1/2, FL/LCC, and FL3A were CD10+ (91%), and 0/46 (0%) expressed IRF4/MUM1. The correlation of CD10 negativity, enhanced IRF4/MUM1 expression and absence of the t(14;18) in FL3 had been noted previously.[8] However, in the study by Karube *et al.*, FL3B and FL3A were not separated and the authors did not specify the distinction of their FL3 from DLBCL/FL3.[8] While they categorized their CD10-IRF4/MUM1+ samples as high-grade FL, including FL3A, FL3B and DLBCL/FL3B, our study clearly demonstrates that a CD10-IRF4/MUM1+ phenotype is infrequent in FL3A, but is a characteristic finding in FL3B

and DLBCL/FL3B. In keeping with the clear association of morphology and immunophenotype, the vast majority of CD10$^-$IRF4/MUM1$^+$ cases in our series (94%) lacked the *BCL2* translocation. Therefore, in contrast to the opinion of Karube *et al.*,[8] the CD10$^-$IRF4/MUM1$^+$ *BCL2* rearrangement-negative samples do not represent a category of FL3 *per se*, but are mainly found in the morphology-based categories of FL3B and DLBCL/FL3B. As expected from their morphologically ambiguous classification, the cases of FL3U in which a clear – and especially reproducible – classification into FL3B or FL3A was not possible, form a mixed category with respect to their immunohistochemical profile, including cases with a CD10$^-$IRF4/MUM1$^+$ phenotype (2 cases) and others with a CD10$^+$IRF4/MUM1$^-$ phenotype (6 cases), and also harbor a considerable proportion BCL2-rearranged cases (6/15, 40%) (*Online Supplementary Table S1*). Since a correlation between IRF4/MUM1 protein expression and translocation of the *IRF4* gene was recently demonstrated in pediatric lymphomas,[18] we investigated the constitution of the *IRF4* gene locus in FL subtypes with or without IRF4/MUM1 expression. However, none of the cases analyzed had a translocation involving *IRF4*. This is likely due to the fact that *IRF4* translocations occur mainly in pediatric or young adult patients (15% *versus* 2% *IRF4* translocations in adult FL patients[18]), while the median age of the patients analyzed for *IRF4* translocations in our sample was 60 years (range, 41 to 80 years; *data not shown*) and, indeed, only one pediatric patient (aged 9 years) was included in this study. Consistent with recent findings, this child with FL3U (ID31, *Online Supplementary Table S1*) did not have a translocation of *BCL2*, showed no BCL2 protein expression,[4] and had a CD10$^-$IRF4/MUM1$^-$ immunophenotype: *IRF4*-translocation analysis was not, however, successful in this particular case.

At present, our data and those from the literature suggest that FL with a significant blast content fall into three categories. FL3A, according to their morphological, immunohistochemical and genetic profiles (centroblasts and centrocytes, CD10 and BCL2 reactivity, frequent *BCL2* rearrangements) resemble FL1/2, as do FL/LCC. On the other hand, DLBCL/FL3B are similar to DLBCL in all aspects. Follicularity in those cases might, therefore, be due to follicular colonization rather than true neo-formation of follicles. FL3B, however, form a currently intermediate group of cases with only low levels of *BCL2* and *BCL6* rearrangements and can, by virtue of this, be distinguished both from the groups of FL1-3A and from DLBCL. Clinical data on this still enigmatic tumor entity remain scarce and are retrospective in nature. They do, however, suggest that the subclassification of FL3A and FL3B is not of clinical relevance, and that, in keeping with this finding, FL3B cannot be cured with at least conventional anthracycline-based chemotherapy protocols, again setting it apart from DLBCL.[13,20] However, in neither of these series were differences in immunophenotype or genetic constitution, documented in our cases, taken into account. On the other hand, one study suggested that it is not the distinction into FL3A or FL3B that is crucial in assessing prognosis, but the question of whether a diffuse component is present or not.[28]

For the time being, it is not clear whether, and if so how, the above findings could be translated into clinical practice. At present, there is certainly no justification for subclassifying FL3 on the basis of immunophenotypic or genetic data, especially because there is still a large overlap in immunophenotypic and genetic features within the cytomorphological groups. Clarification of the question of whether immunophenotypic criteria or genetic data might be used to define prognostically relevant groups must await correlation with clinical data. Notwithstanding this, the in part relatively consistent delineation of immunohistochemical and interphase cytogenetic features in our series of FL suggests that morphologically ambiguous samples of FL3U might - at present - be classified according to their genetic constitution and immunophenotype. For example, FL3U with a CD10$^+$IRF4/MUM1$^-$ phenotype and *BCL2* rearrangement could – for scientific reasons - be tentatively classified as FL3A-related and those FL3U with a CD10$^-$IRF4/MUM1$^+$ immunophenotype lacking *BCL2* translocations may at present be regarded rather as related to FL3B.

Authorship and Disclosures

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