Follicular lymphoma grade 3B and diffuse large B-cell lymphoma present a histopathological and molecular continuum lacking features of progression/transformation

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Abstract

The sole distinguishing feature of follicular lymphoma grade 3B and diffuse large B-cell lymphoma is the growth pattern assessed by histopathology. Diffuse growth defines diffuse large B-cell lymphoma but the clinical relevance of this finding when occurring in follicular lymphoma grade 3B is uncertain. To address this issue, individual and coexisting follicular lymphoma grade 3B and diffuse large B-cell lymphoma were separated and analyzed for immunophenotype and molecular genetic features by fluorescence *in situ* hybridization, targeted sequencing and gene expression profiling. Clinical features of follicular lymphoma grade 3B with and without coexisting diffuse large B-cell lymphoma were studied in homogeneously treated patients from a prospective randomized trial. Follicular lymphoma grade 3B and diffuse large B-cell lymphoma frequently show an intermediate growth pattern and/or occur simultaneously in the same tissue at the time of initial diagnosis. When occurring simultaneously follicular lymphoma grade 3B and diffuse large B-cell lymphoma do not differ significantly for genetic aberrations or phenotype but have distinct gene expression features reflecting a divergent microenvironment. Follicular lymphoma grade 3B with and without coexisting diffuse large B-cell lymphoma do not differ for major clinical parameters such as International Prognostic Index, response to immuno-chemotherapy, progression or overall survival. Follicular lymphoma grade 3B and simultaneous diffuse large B-cell lymphoma are molecularly homogenous. Histological detection of diffuse large B-cell lymphoma is not associated with features of a more aggressive disease and does not reflect transformation or progression of follicular lymphoma grade 3B.

Introduction

Follicular lymphoma (FL) is one of the most frequent lymphoma entities in central Europe and Northern America and is subdivided into "grades" by morphology.[1] Follicular lymphoma grades 1, 2 and 3A are composed of centrocytes and centroblasts and distinguished by centroblast content whereas FL grade 3B (FL3B) consists exclusively of centroblasts.[1] Since the cytomorphology of FL3B is identical to that of diffuse large B-cell lymphoma (DLBCL), the sole distinguishing feature of FL3B and DLBCL is the growth pattern assessed by histopathology which is follicular in FL3B and diffuse in DLBCL. Once the neoplastic cells in FL3B display areas of diffuse growth the lymphoma fulfills the diagnostic criteria of DLBCL according to the World Health Organization (WHO) classification.[1] Thus, diffuse areas of FL3B are considered "transformation" and this feature has been suggested to be of clinical significance.[2,3] Unlike FL grades 1, 2 and 3A, FL3B is considered an aggressive lymphoma by most clinical research groups and treated in the same way as DLBCL[4] despite the fact that under rituximab-containing poly-chemotherapy (e.g., rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone [R-CHOP]) outcomes might not be very different between FL3A and FL3B.[5,6] Transformation of FL grades 1, 2 and 3A to DLBCL has been shown to be associated with major changes in molecular pathogenic pathways and is well established to reflect a true "transformation" with change in clinical behavior and

necessity of treatment strategies that may differ from those for FL 1, 2 and 3A.[7] In a large retrospective study FL3B seemed to be associated with a favorable outcome.[8] However, following the current definition of the WHO classification FL3B harboring areas of diffuse growth fulfilling the criteria of DLBCL are rather rare and probably underrepresented[5,9] or even excluded from analysis in more recent studies on the clinical features of FL3B.[6,8] It is, therefore, unclear whether histological transition of follicular to diffuse growth reflects a clinically relevant transformation at all. Lack of knowledge regarding the biological and clinical significance of transformation of FL3B may however create a considerable clinical problem since FL3B occurs in patients who are often young - a subgroup of patients who warrant careful consideration of therapy intensity with respect to long-term toxicity.[10]

As FL3B is one of the rarest grades of FL very few molecular studies have been conducted and most of them focused on the distinction of FL3B from other grades and DLBCL.[11-14] In the current study we aimed to understand the molecular and clinical features of FL3B showing transition into DLBCL in order to provide a definition of "transformation" in FL3B guiding clinical decision-making.

Methods

Case selection, histological evaluation and immunohistochemistry

Cases with a diagnosis of FL3B with or without a DLBCL component diagnosed between 2012 and 2016 at the Hematopathology Section and Lymph Node Registry of the University Hospital Schleswig-Holstein, (Kiel, Germany) were identified in the files and re-evaluated with regard to diagnosis and growth patterns as well as suitability for molecular analysis. In total, 51 specimens with a histological picture of FL3B were identified, two of which were excluded later as they turned out to have an *IRF4* break and these are considered a separate entity by the WHO classification.[1] Staining for CD20 (clone L26, DAKO), CD10 (clone 56C6, Novocastra), Mum1 (clone MUM1P, Dako), Bcl6 (clone BL6.02, DCS) and Bcl2 (clone 100/D5, DBS) was evaluated semiquantitatively (negative, <25% of lymphoma cells positive, <50% positive, <75% positive, >75% positive) and proliferative rate was determined according to Ki67 (clone SP6, Neomarkers) in steps of 10% by visual inspection. Immunohistochemical classification of the cell of origin of DLBCL components was performed applying the Hans classifier.[15] Areas of defined growth patterns were selected for tissue microarray construction in 47 cases with sufficient material. Meshworks of follicular dendritic cells were evaluated in tissue microarrays for each growth pattern semiquantitatively by CD21 staining (0 = absence of meshworks, 1 = markedly reduced covering <50% of follicles, 2 = slightly reduced covering >50% of follicles and 3 = intact covering whole area of follicle; clone 2G9, Novocastra). Punches with a tissue microarray needle (1 mm diameter) from each area were also used for nucleic acid extraction. To prevent cross-contamination of DNA/RNA analytes the tissue microarray needle was punched three to five times in an empty paraffin block between each punching of a patient's specimen.

The study was conducted in accordance with the recommendations of the ethics board of the Medical Faculty, University of Kiel (D447/10) for the use of archival tissue specimens. Informed consent was obtained from patients treated in the PETAL trial (see below).

Fluorescence *in situ* hybridization

Fluorescence i*n situ* hybridization (FISH) was performed on 5 μm slides from the tissue microarrays, allowing separate analysis of different growth patterns. Probes for *IRF4*, *BCL2*, *BCL6* and *MYC* were obtained from ZytoVision (Bremerhaven, Germany) and applied as previously described.[16]

DNA and RNA extraction

Cases for molecular genetic analyses were chosen when the tissue quality was assumed to be sufficient for nucleic acid extraction. Simultaneous DNA and RNA extraction was done using the AllPrep DNA/RNA FFPE Kit (Qiagen) according to the manufacturer´s instructions. For each patient different growth patterns were extracted separately from tissue microarray needle biopsies taken from the respective areas (2-3 biopsies per specimen).

Mutation analyses

Mutation analyses were done on DNA isolated from the formalin-fixed paraffin-embedded material of the diagnostic biopsy using an AmpliSeq Custom DNA Panel covering genes or mutational hotspots known to be recurrently mutated in aggressive B-cell lymphomas (*Online Supplementary Table S1*). Libraries were prepared according to the manufacturer's instructions and sequencing was performed on a MiSeq instrument using V3 sequencing chemistry.

FASTQ data were analyzed using JSI SeqNext software (JSI Medical Systems GmbH, Ettenheim, Germany). Formalin-fixed paraffin-embedded tissue from reactive lymph nodes from eight healthy individuals (a cancer infiltration was excluded by expert hematopathologists) was used as control tissue, and sequence alterations identified in those samples were subtracted from those of the lymphoma patients. Furthermore, common single nucleotide polymorphisms with ≥1% variant allele frequency were excluded. Potential protein changing alterations with at least 15% variant allele fraction were analyzed in more detail using the ENSEMBL variant effect predictor (*http://www.ensembl.org/Homo_sapiens/Tools/VEP*). However, as the library preparation process contains polymerase chain reaction amplification

steps to enrich the target regions no interpretation of the variant allele fractions was possible. Furthermore, no germline material was available from those samples in order to be able to differentiate between somatic and germline variants. To overcome this limitation, variants were categorized based on the variant effect prediction by SIFT, PolyPhen, FATHMM and Condel into six different groups: (i) high impact; (ii) moderate impact and deleterious or damaging effect; (iii) moderate impact but mixed effect prediction (deleterious/damaging or tolerated based on the different tools used); (iv) moderate impact and tolerated or benign; (v) moderate impact but no additional information; and (vi) low impact. Variants with high impact, moderate/deleterious effect prediction and moderate/mixed effect prediction were considered to affect protein function negatively.

Gene expression analysis

Gene expression was analyzed as previously described applying NanoString technology and the PanCancer Immune Profiling Panel.[17] Background thresholding and normalization were performed by the NSolver software (version 4.0; NanoString Technologies). Twenty housekeeping genes were chosen for normalization in a two-step process: (i) housekeeping genes with average counts <100 were excluded; and (ii) 20 housekeeping genes with the lowest count variability were chosen for normalization. Endogenous genes with normalized expression levels below the calculated background threshold in >20% of the analyzed specimens were excluded from further analysis. Fold changes between follicular and diffuse growth patterns were calculated for each individual case. Genes with a fold change of >1.2 or <-1.2 in at least 50% of cases were chosen and mean fold changes of these genes were calculated as fold change of the geometric means of the respective single case gene expression levels. Genes with a mean fold change of >1.5 or <-1.5 were considered to be differentially expressed.

Clinical analysis

The features of patients with FL3B (n=17) and FL3B+DLBCL (n=16) were analyzed in patients treated in the prospective randomized 'Positron Emission Tomography-Guided Therapy of Aggressive Non-Hodgkin Lymphomas' trial (PETAL: clinicaltrials.gov NCT00554164 and EudraCT 2006-001641-33).[18] Patients received two cycles of standard R-CHOP followed by interim [^{18}F]fluorodeoxy-glucose-positron emission tomography (PET). Interim PET-negative patients continued R-CHOP whereas interim PET-positive patients were randomized between continued R-CHOP and an intensive methotrexate- and cytarabine-based Burkitt lymphoma protocol. According to current treatment guidelines[19] all patients were treated at the timepoint of first diagnosis.

Statistical analysis

Statistical analysis was performed using GraphPadPrism (version 7.00 for Windows, GraphPad software, La Jolla, CA, USA) applying the χ^2 test, Fisher exact test and unpaired *t*-test as indicated in the Results section. Kaplan-Meier curves were generated using SPSS statistics (version 26.0, IBM, Armonk, NY, USA).

Results

Growth pattern of follicular lymphoma grade 3B with a continuum to diffuse large B-cell lymphoma

Forty-nine lymphomas with a diagnosis FL3B were reanalyzed for growth pattern. All cases fulfilled the criteria of FL3B and 27/49 (55%) additionally harbored areas with diffuse growth representing DLBCL by definition (FL3B+DLBCL). None of the cases included any low-grade FL component. None of the cases showed the typical constellation of pediatric type FL according to the current WHO classification.[1] Relapse biopsies were available for two patients both of whom had FL3B+DLBCL at primary diagnosis and DLBCL at relapse. The proportion of DLBCL ranged between 10% and 95% in individual cases (mean: 49%). The cytomorphology of the DLBCL component was centroblastic in 25/27 cases and immunoblastic in 2/27 cases. In all cases, the morphology of the DLBCL component matched the morphology of the FL3B component. Within the areas of follicular growth two different growth patterns were noticed: (i) clearly demarcated, roundish follicles that were separated from each other (follicular pattern) (Figure 1) and (ii) follicles localized close to each other appearing to merge (confluent pattern) (Figure 1). The confluent pattern was clearly distinguishable from a diffuse pattern/DLBCL in hematoxylin and eosin staining and could be further highlighted by staining for T cells and follicular dendritic cells (Figure 1). Of the 22 pure FL3B lacking an additional DLBCL component, only four cases showed a pure follicular pattern (4/22, 18%), whereas 11/22 (50%) cases solely displayed the confluent pattern. A combination of both patterns was detectable in 7/22 (32%) of pure FL3B. The distribution of these growth patterns within the FL3B component was different in FL3B+DLBCL cases (confluent pattern 14/27 [52%], follicular pattern 7/27 [26%] and a combination of both patterns 6/27 [22%], P=0.0421, χ^2 test) although the differences must be interpreted with caution because of the small number of cases. All growth patterns and their combinations are indicated in Table 1.

Homogeneous immunophenotype in follicular lymphoma grade 3B and diffuse large B-cell lymphoma

CD20 was homogeneously expressed in all 49 cases. CD10 expression was more variable with 22/49 cases (45%) being completely negative, 4/49 cases (8%) with ex-

pression in <50% of cells and 23/49 cases (47%) with expression in >75% of cells. Bcl2 staining was available for 48 cases. Bcl2 was completely negative in 5/48 cases (10%), positive in <50% of cells in 7/48 cases (15%) and positive in >50% of cells in 36/48 cases (75%). Staining for Mum1 was done in 36 cases. Mum1 was completely negative in 3/36 cases (8%), positive in <25% of cells in 9/36 cases (25%), positive in 25% to <50% of cells in 4/36 cases (11%), positive in 50% to <75% of cells in 11/36 cases (31%) and positive in ≥75% of cells in 9/36 cases (25%). Bcl6 staining was available for 29 cases. Of these, seven cases (24%) were positive in 50% to <75% of cells and 22 cases (76%) were positive in ≥75% of cells. The proliferative rate by Ki67 ranged between 30% and 90% with a mean proliferative rate of 63% in the whole cohort. Classification of the cell of origin was available for 26 cases with a DLBCL component. Of these, 19 cases (73%) displayed a germinal center B-cell-phenotype and seven cases (27%) displayed a non-germinal center B-cell-phenotype according to the Hans classifier.[15] Despite intertumoral variability, expression of the above-mentioned antigens did not differ between different growth patterns within an individual patient/lymphoma. Of note, FL3B and DLBCL areas in the same specimen showed the same proliferative rate according to Ki67 (Figure 2). Comparing FL3B and FL3B+DLBCL, no significant differences between mean proliferative rates were detectable (59% and 65%, respectively, P=0.1349, unpaired t-test). CD21 staining was evaluable in 12 follicular FL3B components, 26 confluent FL3B components and 16 DLBCL components of 34 cases. Meshworks of follicular dendritic cells showed considerable intercase variability. The highest scores were reached in follicular FL3B (8/12, 67% slightly reduced/intact and 4/12, 33% markedly reduced); DLBCL components mostly showed markedly reduced or absent meshworks (1/16, 6% slightly reduced and 15/16, 94% markedly reduced/absent). Confluent FL3B presented intermediate stages of follicular dendritic cell preservation (14/26, 54% slightly reduced/intact and 12/26, 46% markedly reduced/absent meshworks), supporting the idea that confluent FL3B represents an intermediate stage between follicular FL3B and DLBCL. Within individual cases with evaluable FL3B and DLBCL components (n=14), a reduction of meshworks could be observed between the FL3B and DLBCL components in 9/14 cases (64%) and the same stage of preservation in 5/14 cases (36%).

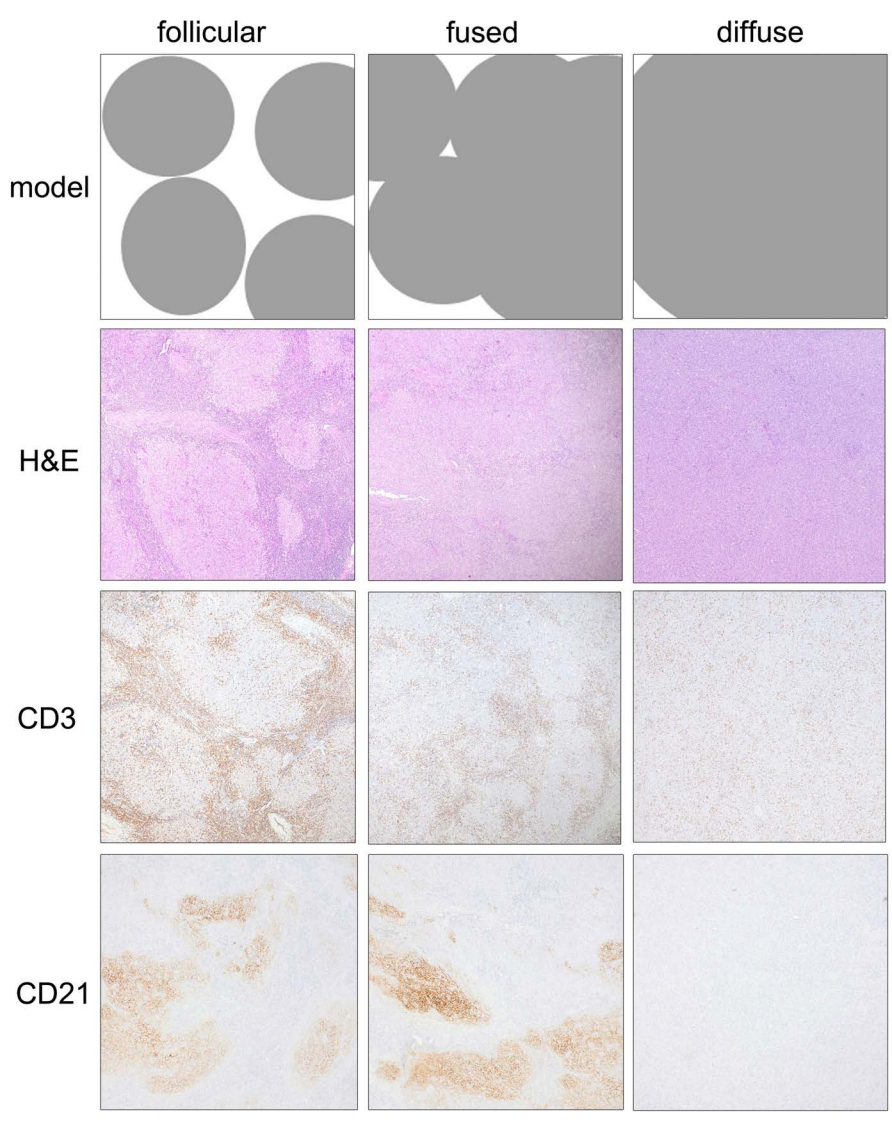

Figure 1. Growth pattern of follicular lymphoma grade 3B and diffuse large B-cell lymphoma. The three patterns observed are shown for exemplary cases in the three columns as a scheme/model, hematoxylin and eosin staining (H&E) and staining for CD3 and CD21. Original magnification 40x.

Chromosomal aberrations are mostly stable between follicular lymphoma grade 3B and diffuse large B-cell lymphoma

Lymphomas with breaks in *IRF4* were excluded since these are considered a distinct entity in the WHO classification. FISH to detect breaks in *BCL2*, *BCL6* and *MYC* was performed for 40 cases, 21 of which contained a DLBCL component. In any lymphoma containing multiple growth patterns these were analyzed separately. Overall, 27 chromosomal breaks were detectable in 25 cases and 15 cases did not show breaks in the above-mentioned genes. One case presented a double-hit constellation with *MYC* and *BCL6* rearrangements (FL3B with confluent pattern). Chromosomal breaks occurred at a similar frequency in FL3B and

Table 1. Coexistence of growth patterns in follicular lymphoma grade 3B.

	Follicular	Confluent	Diffuse	Total
FL3B (N=22)	+	-	-	4/22 (18%)
	-	+	-	11/22 (50%)
	+	+	-	7/22 (32%)
FL3B/DLBCL (N=27)	+	-	+	7/27 (26%)
	-	+	+	14/27 (52%)
	+	+	+	6/27 (22%)

Numbers and percentages correspond to the respective subgroup. FL3B: follicular lymphoma grade 3B; DLBCL: diffuse large B-cell lymphoma.

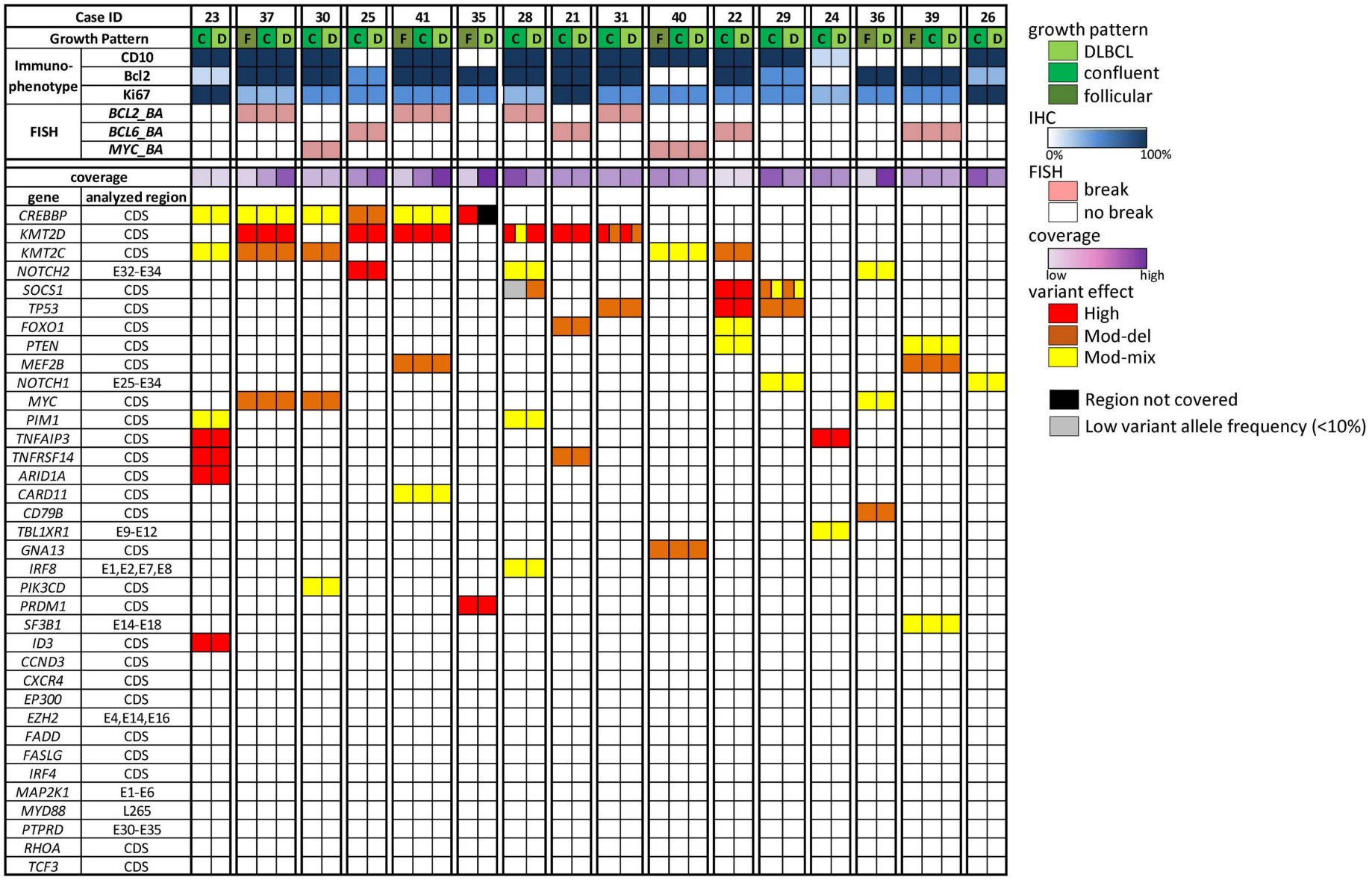

Figure 2. Mutational pattern of the different growth patterns of follicular lymphoma grade 3B + diffuse large B-cell lymphoma. Within each lymphoma the patterns were analyzed separately. Individual cases are indicated by the case identifie (ID) and growth pattern as D: diffuse large B-cell lymphoma (DLBCL) component; F: follicular component; C: confluent component. The immunophenotype was scored in a five-tiered way 0%; 1-25%; 26-50%; 51-75%; >75% positive tumor cells and is displayed as a color code as shown in the scale bar. Fluorescence *in situ* hybridization (FISH) was done using break apart probes (BA). Potential protein-changing variants identified in the different components were colored according to the variant effect prediction (red: high; dark orange: moderate, deleterious; yellow: moderate, deleterious and tolerated effect based on different algorithms used, black: region was not covered in the DLBCL sample, gray: variant was identified with low variant allele frequency (<10%) in the confluent component. If more than one variant with different effects targeting one gene was identified in one component both variant effects are shown separately. E: exon; CDS: coding region; #: region was not covered in the DLBCL component of that sample. $: variant was identified with low (<10%) variant allele frequency in the confluent component. IHC: immunohistochemistry.

FL3B+DLBCL (FL3B: *BCL2* in 2/19 [11%], *BCL6* in 8/19 [42%], *MYC* in 1/19 [5%]; FL3B+DLBCL: *BCL2* in 6/21 [29%], *BCL6* in 7/21 [33%], *MYC* in 3/21 [14%], *P*=0.2409 for *BCL2*, *P*>0.9999 for *BCL6*, *P*=0.6094 for *MYC*, Fisher exact test). FISH results for at least two growth patterns of the same lymphoma were available for 21 cases. In most cases the chromosomal aberrations were shared between the different growth patterns (20/21 cases, 95%). However, in 1/21 (5%) cases a divergent result was observed concerning *BCL6*. This case harbored a *BCL6* break in the confluent area but not in the follicular areas of a FL3B (*data not shown*).

The microenvironmental composition of follicular lymphoma grade 3B and diffuse large B-cell lymphoma differs

In order to understand whether the growth patterns represent molecular progression/evolution of the disease, gene expression was analyzed in FL3B+DLBCL cases. Assuming that the follicular pattern and DLBCL represent the two ends of a spectrum of growth patterns in each individual lymphoma, these areas were analyzed separately in cases with both components available (n=6). Since DLBCL are known to be a heterogeneous disease with their molecular features differing from patient to patient, we analyzed differential expression within each patient comparing the follicular FL3B with the DLBCL component in cases with both components available. Using the criteria described in the Methods section we identified a low number of genes (n=45) differentially expressed in multiple patients (*Online Supplementary Figure S1*). Of these, 33/45 (73%) genes were more highly expressed in the follicular FL3B area and reflected the expression pat-

tern of the germinal center microenvironment, such as follicular dendritic cells (*CD21*), follicular T helper cells (*ICOS*, *STAT4*), T cells (*CD3*, *TCF7*, *TXK*) and *CXCL12*, which has previously been described to be upregulated in FL stromal cells.[20] Furthermore, genes more highly expressed in follicular areas reflected a greater abundance of cells of innate immunity (*C1QB*, *SIGLEC1*, *TPSAB1*) including natural killer cells (*KLRB1*, *KLRG1*) compared to the DLBCL component. In line with a higher content of immune cells, genes involved in trafficking e.g. by adhesion and motility, were more highly expressed in follicular areas (*CCR7* and its ligand *CCL21*, *CDH1*, *ITGA6*, *ITGB6*). Alterations of the extracellular matrix between follicular and diffuse areas are reflected by differential expression of collagen III α and fibronectin which were more highly expressed in the DLBCL component. Of note, two of 12 genes more highly expressed in the diffuse part are known to be involved in T-cell activation (*CD70* and *TNFSF4/OXL40*). Only a few of the differentially expressed genes could be assigned to B-cell differentiation (e.g. *CD27*) suggesting that the gene expression pattern of the neoplastic B cells does not differ substantially between follicular (FL3B) and diffuse (DLBCL) areas. No differences were observed in genes described to be associated with transformation or an aggressive phenotype of FL (*MYC*, *NOTCH*, NFkB signaling pathway genes, data not shown).

Molecular genetic features are stable between follicular lymphoma grade 3B and diffuse large B-cell lymphoma

Genes recurrently mutated in mature B-cell lymphoma were analyzed by targeted sequencing in pure FL3B (n=11) and FL3B+DLBCL (n=16). The FL3B+DLBCL group consisted of two cases containing follicular and diffuse components, ten cases with a confluent and a diffuse component and four cases with all growth patterns in the same specimen. Areas representing growth patterns were separated by macrodissection and analyzed individually (yielding 36 sequencing results). Sequencing retrieved a mean read depth of 4,258 (min 276, max 10,131). Overall, 81 genetic variants were detected (*Online Supplementary Table S2*). The genetic variants were mostly missense variants (n=56), followed by nonsense (n=9), and splice site or splice region variants (n=8), frameshift (n=4) and inframe variants (n=4). The pure FL3B group consisted of nine cases with a confluent growth pattern, one case with a follicular growth pattern and one case with a follicular and confluent growth pattern of which only the confluent component was analyzed. Sequencing retrieved a mean read depth of 5,146 (min 967, max 9,769). Overall, 22 genetic variants were detected (*Online Supplementary Table S2*). The genetic variants were missense in most cases (n=14), followed by nonsense (n=7) and splice site or splice region variants (n=1). Since germline DNA was not available, variant effect prediction using ENSEMBLE was performed to estimate the functional relevance of the genetic alterations. In the FL3B+DLBCL group, variant effect prediction identified 16/81 (20%) variants with high impact, 17/81 (21%) with moderate/deleterious effects, 29/81 (36%) with moderate/mixed effects, 13/81 (16%) with moderate/tolerated or unclear effects and 6/81 (7%) with low impact. In the pure FL3B group, 9/22 (41%) variants were predicted to have high impact, 6/22 (27%) with moderate/deleterious effects, 4/22 (18%) with moderate/mixed effects and 3/22 (14%) with moderate/tolerated effects. The number of variants detected per case was significantly different between pure FL3B (0 to 6; mean 2) and FL3B+DLBCL (1 to 11; mean 5.1; P=0.0037, unpaired t-test). Overall, 62/81 (77%) variants in FL3B+DLBCL and 19/22 (86%) variants in pure FL3B were predicted to affect protein function negatively. Considering only variants with potentially negative effects on protein function, significant differences between FL3B and FL3B+DLBCL could still be observed (FL3B: 0 to 6 variants per case, mean 1.7 and FL3B+DLBCL: 1 to 8 variants per case, mean 3.9; P=0.0091, unpaired t-test). However, as a targeted sequencing approach was applied, this does not allow conclusions on the whole load of genetic variants in individual tumors. The most frequently altered genes with potentially negative effects on protein function were *KMT2D* (6/16 cases, 38%), *CREBBP* (6/16 cases, 38%) and *KMT2C* (5/16 cases, 31%) in FL3B+DLBCL and *TP53* (3/11 cases, 27%), *MYD88* (2/11 cases, 18%) and *KMT2D* (2/11 cases, 18%) in pure FL3B. No significant differences were found between FL3B and FL3B+DLBCL considering variants in specific genes (*Online Supplementary Table S4*).

In 14/16 (88%) cases of FL3B+DLBCL all variants were shared between the different specimens/areas of the corresponding sample. Thus, overall 77/81 (95%) variants did not differ between FL3B and DLBCL occurring in the same patient (Figure 2). In only two cases, four variants were detected that differed between FL and DLBCL within the same patient. In one sample (case 35) a variant in the *CREBBP* gene was detected in the follicular component but no sequencing result was obtained for the DLBCL component due to low coverage. In the other sample (case 28), three variants were discrepant between the FL3B (confluent pattern) and the DLBCL component. Two variants affecting *NOTCH1* and *SOCS1* were identified in the confluent and the DLBCL component, respectively; both had a very low frequency (<10% of reads) in the other component and were therefore initially not considered. In contrast, a *KMT2D* mutation was present in the FL3B and completely absent in the DLBCL component (Figure 2). This variant was predicted to have a moderate/mixed effect.

The clinical features of follicular lymphoma grade 3B alone and with diffuse large B-cell lymphoma do not differ

To understand whether the co-occurrence of DLBCL with

FL3B is associated with clinical progression, we analyzed the subgroup of FL3B in the prospective randomized PETAL trial.[18] In this clinical cohort 17 patients were classified as having FL3B and 16 as having FL3B+DLBCL. All the patients with FL3B and 15/16 of those with FL3B+DLBCL had a favorable interim PET scan and were treated with six cycles of R-CHOP, while one FL3B+DLBCL patient had an unfavorable interim PET scan and received two cycles of R-CHOP followed by the Burkitt lymphoma protocol. The presence of the confluent pattern described in the current manuscript was not assessed by central pathology review in PETAL and is thus not available for further analysis. The baseline characteristics of the FL3B and FL3B+DLBCL cohorts did not differ for parameters known to be associated with aggressive disease such as advanced stage, increased lactate dehydrogenase level or International Prognostic Index (Table 2, comparison with DLBCL treated in PETAL in *Online Supplementary Table S3*). Moreover, we did not find any significant difference in overall treatment response and overall survival (Table 2). As a consequence, progression-free survival and overall survival did not differ between FL3B and FL3B+DLBCL (Figure 3).

Discussion

FL3B is a rare subtype of FL, underrepresented in clinical and translational research studies.[21] The definition of FL3B is exclusively based on histological features: (i) differentiation arrest of neoplastic germinal center cells as centroblasts leading to absence of centrocytes and (ii) follicular growth. The latter is the sole feature distinguishing FL3B from DLBCL. Follicular growth describes an arrangement of lymphoma cells in roundish accumulations of cells which contain microenvironmental structures of physiological germinal centers such as follicular dendritic cells, follicular T-helper cells and occasionally follicle mantle cells.[22] These organoid arrangements resemble physiological germinal centers more closely in low-grade FL such as

Table 2. Clinical characteristics and treatment response of follicular lymphoma grade 3B with or without diffuse large B-cell lymphoma in patients treated in the prospective randomized 'Positron Emission Tomography-Guided Therapy of Aggressive Non-Hodgkin Lymphomas' (PETAL) trial.[18]

	FL3B alone		FL3B with DLBCL		
Number of patients	17		16		
Median age (range)	51 years (29-72)		57 years (29-76)		
Baseline characteristics	**Number**	**Percent**	**Number**	**Percent**	***P***
Male sex	13	76.5	7	43.8	0.0799
Age ≥60 years	5	29.4	6	37.5	0.7207
ECOG performance status >1	1	5.9	0	0.0	>0.9999
Lactate dehydrogenase >ULN	10	58.8	7	43.8	0.4935
Ann Arbor stage III or IV	11	64.7	10	62.5	>0.9999
Extranodal manifestations >1	2	11.8	3	18.8	0.6562
Bone marrow infiltration	1	5.9	0	0.0	>0.9999
B symptoms	5	29.4	1	6.3	0.1748
International Prognostic Index			0.7673		
Low	9	53.0	8	50.0	
Low-intermediate	3	17.6	3	18.8	
High-intermediate	4	23.5	5	31.2	
High	1	5.9	0	0.0	
Treatment response					
Overall response	16	94.1	15	93.8	>0.9999
Complete remission	12	70.6	10	62.5	>0.9999

P-values according to the Fisher exact test and for the International Prognostic Index by the χ^2 test. FL3B: follicular lymphoma grade 3B; DLBCL: diffuse large B-cell lymphoma; ECOG:, Eastern Cooperative Oncology Group; ULN, upper limit of normal.

FL grades 1 and 2 compared to FL grade 3A and FL3B. Initially, the grades of FL were thought to reflect a multistep process of tumor progression in analogy to grades of differentiation in solid tumors.[23] However, there are several findings suggesting that at least a large fraction of FL3B cases are not a progressive form of FL grades 1, 2 and 3A. First, unlike FL grades 1, 2 and 3A, FL3B is diagnosed more frequently in younger patients, suggesting a unique pathogenesis.[10,21] Second, molecular studies, e.g. on chromosomal translocations show, a much lower frequency of aberrations involving *BCL2* and a higher frequency of *BCL6* translocations in FL3B than in FL grades 1, 2, and 3A.[9,11,13,24] Third, FL3B often co-exists with a DLBCL at initial diagnosis whereas transformation of indolent FL grades 1, 2 and 3A mostly occurs at a later stage of the disease.[5,9] Fourth, FL3B rarely follows or co-exists with FL grades 1, 2 and 3A, again suggesting differences in pathogenic pathways.[9]

In the current study we analyzed an aspect of FL3B biology not addressed by previous studies. We aimed to understand whether the loss of follicular growth indicates molecular and clinical progression/transformation. To the best of our knowledge, this is the first study specifically analyzing the intratumoral heterogeneity of FL3B and

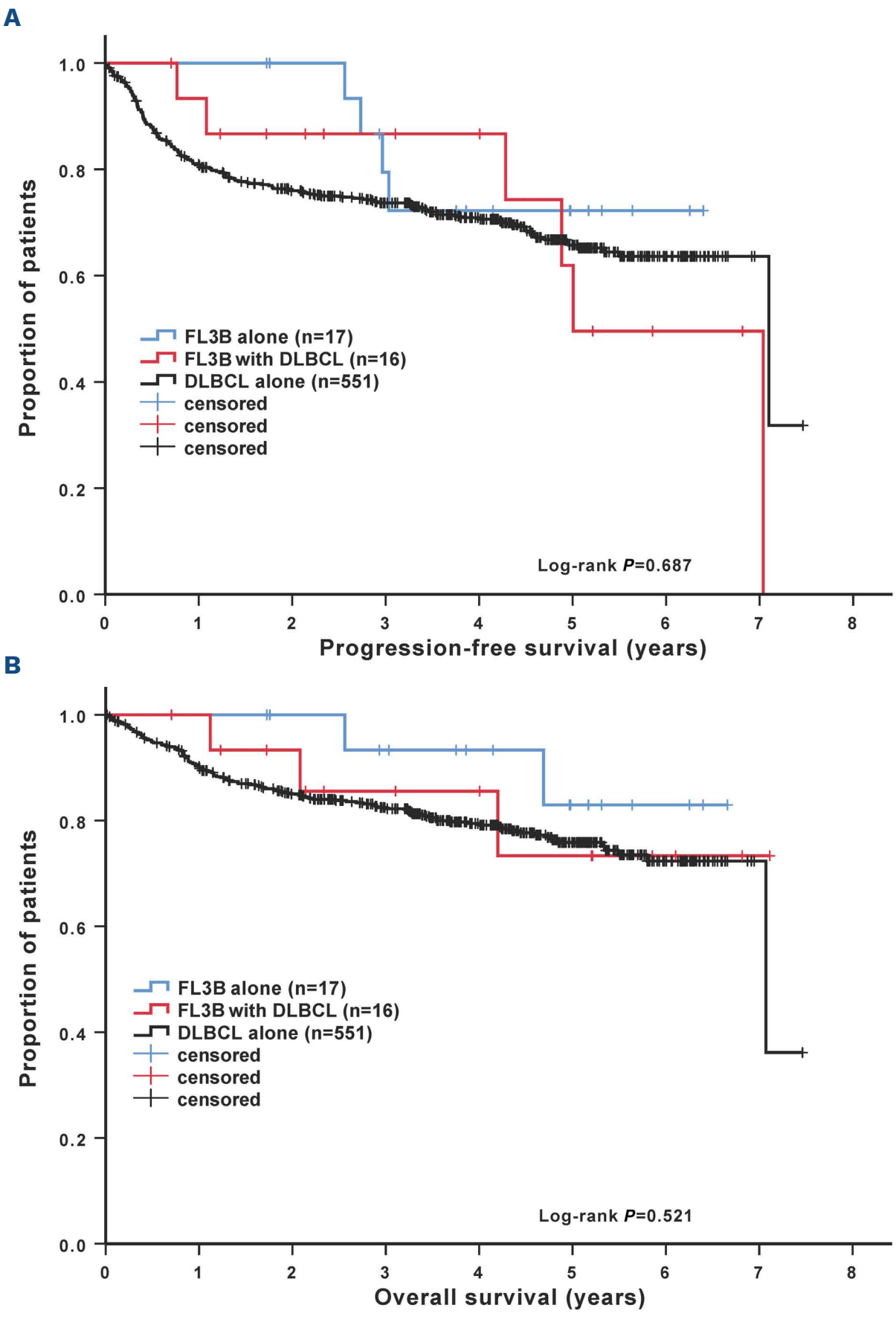

Figure 3. Kaplan-Meier survival estimates for progression free (A) and overall survival (B) of patients with follicular lymphoma grade 3B with or without diffuse large B-cell lymphoma. Patients with diffuse large B-cell lymphoma (DLBCL) treated in the same trial are shown as a control (see *Online Supplementary Table S3*). As patients with activated B-cell or germinal center B-cell DLBCL did not differ significantly regarding progression-free survival and overall survival these subgroups were not differentiated.

DLBCL within individual patients/lymphomas. Similarly to previous studies,[5,9] we found that a large number of cases of FL3B were associated with DLBCL at first diagnosis. Moreover, we found a pattern intermediate between follicular and diffuse growth endorsing the impression of a pathological continuum between FL3B and DLBCL. Furthermore, we showed that the molecular features of co-existing FL3B and DLBCL were homogeneous and that there was no molecular evidence of progression/transformation. In fact, the differences between follicular and diffuse growth - representing FL3B and DLBCL, respectively – were subtle and restricted to the features of the non-neoplastic microenvironment. Considering these findings it was not surprising to see that clinical features did not differ between patients with FL3B and those with FL3B+DLBCL. It seems reasonable to assume that FL3B represents a molecular and clinical continuum with DLBCL. Thus, FL3B+DLBCL does not reflect progression or transformation of the disease, suggesting that treatment should follow the same guidelines for FL3B and DLBCL. Of note, the subtype of *IRF4* translocation-positive lymphomas now recognized by the WHO classification was excluded from our analysis. However, large B-cell lymphoma with *IRF4* translocations shares features with FL3B such as histology (mostly presenting as FL3B and/or DLBCL),[25] patients' age (younger adults)[10,26] and outcome (favorable).[27] To what extent FL3B lacking *IRF4* aberrations and large B-cell lymphoma with *IRF4* translocations may in fact share pathogenic features needs to be addressed in future studies that might help to specify diagnostic criteria. Another interesting group of patients to analyze in the future might be those with FL3B at primary diagnosis and DLBCL at relapse. However, this is hindered by the fact that only a small proportion of patients with FL3B relapse and of those who do only few undergo a second biopsy. This also holds true for our cohort, in which a second biopsy was only available for two patients. Moreover, a more detailed molecular analysis of FL3B and FL3B+DLBCL, such as that applied in our current study, may provide a molecular genetic definition of FL3B independent of the histological detection of follicular growth. One could speculate that a molecular definition of FL3B might identify a molecular counterpart among DLBCL lacking a histopathologically detectable FL3B component but sharing clinical features.

Disclosures

AH has received travel expenses from Celgene and Roche. UD has received research funding from Amgen; has received honoraria and research funding from Celgen and Roche; has acted as a consultant for and received honoraria from AbbVie and Gilead; and has received honoria from Janssen. WK has received honoraria and research funding from Amgen, Regeneron, Hoffman-La Roche and Takeda.

Contributions

KK and WK designed the research project, KK, CH and JR generated and analyzed pathological and molecular data, AH and UD analyzed clinical data. KK, JR and WK wrote the manuscript. All authors read and agreed on the final version of the manuscript.

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Data-sharing statement

Data will be shared according to ethical and administrative guidelines upon request for collaboration.

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