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Gene expression profiling reveals a close relationship between follicular lymphoma grade 3A and 3B, but distinct profiles of follicular lymphoma grade 1 and 2

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ABSTRACT

A linear progression model of follicular lymphomas (FL) FL1, FL2 and FL3A has been favored, since FL3A often co-exist with an FL1/2 component. FL3B, in contrast, is thought to be more closely related to diffuse large B-cell lymphoma (DLBCL), and both are often simultaneously present in one tumor (DLBCL/FL3B). To obtain more detailed insights into follicular lymphoma progression, a comprehensive analysis of a well-defined set of FL1/2 (n=22), FL3A (n=16), FL3B (n=6), DLBCL/FL3B (n=9), and germinal center B-cell-type diffuse large B-cell lymphoma (n=45) was undertaken using gene expression profiling, immunohistochemical stainings and genetic analyses by fluorescence *in situ* hybridization. While immunohistochemical (CD10, IRF4/MUM1, Ki67, BCL2, BCL6) and genetic profiles (translocations of BCL2, BCL6 and MYC) delineate FL1-3A from FL3B and DLBCL/FL3B, significant differences were observed between FL1/2 and FL3A upon gene expression profiling. Interestingly, FL3B turned out to be closely related to FL3A, not categorizing within a separate gene expression cluster, and both FL3A and FL3B showed overlapping profiles in between FL1/2 and diffuse large B-cell lymphoma. Finally, based upon their gene expression pattern, DLBCL/FL3B represent a composite form of FL3B and DLBCL, with the majority of samples more closely resembling the latter. The fact that gene expression profiling clearly separated FL1/2 from both FL3A and FL3B suggests a closer biological relationship between the latter. This notion, however, is in contrast to immunohistochemical and genetic profiles of the different histological FL subtypes that point to a closer relationship between FL1/2 and FL3A, and separates them from FL3B.

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Introduction

Follicular lymphoma (FL) comprises approximately 30% of B-cell non-Hodgkin lymphomas (B-NHL) and represents the most common type of indolent B-NHL. FL originates from germinal center B cells (GCB) characterized by expression of CD10 and BCL6. FL consists of a mixture of centroblasts and centrocytes, the relative ratio of which determines the histological grade. While FL1/2 and FL3A consist of centrocytes and centroblasts (the difference between them being the num-

ber of centroblasts), FL3B only harbors centroblasts and centrocytes are not present.¹ Although criteria for the histological grading of FL are well-defined,^{1,2} its precise assessment is challenging even for expert hematopathologists, in some cases resulting in interobserver variability in daily routine diagnostics.^{3,4} On the genetic level, approximately 85% of FL are characterized by the hallmark translocation t(14;18)(q32;q21), resulting in the juxtaposition of *BCL2* to the *IGH* gene locus and, subsequently, to constitutive overexpression of *BCL2* and inhibition of apoptosis. The evolution of novel cell clones with modified growth potential, morphologically often characterized by a higher number of centroblasts and/or by an increased proliferation index, is characteristic of progression of FL.² Approximately 30% of FL transform to a more aggressive lymphoma, usually diffuse large B-cell lymphoma (DLBCL), which is typically associated with inferior clinical outcome.⁵ However, the genetic mechanisms underlying the progression and transformation of FL are poorly understood.

Since FL grade 3A often co-exist with an FL1/2 component, and harbor the t(14;18) in approximately 60% of cases, a linear progression model of FL1, FL2 and FL3A has been developed, although FL3A does not necessarily evolve from FL1/2.³ FL3B, on the other hand, is presumed to be more closely related to DLBCL, and both FL3B and DLBCL are often simultaneously present in a lymph node.^{2,6,7} Although a molecular characterization of FL3A and FL3B versus FL1/2 has been attempted in the past, many reports have only addressed either immunohistochemical and/or genetic differences.^{6,8,9} The main goal of the present study, therefore, was the comprehensive genetic analysis of a well-defined set of FL3A and FL3B and their comparison with related entities such as GCB-type DLBCL and DLBCL with an FL3B component by gene expression profiling, immunohistochemistry, and genetic analysis by fluorescence *in situ* hybridization (FISH).

Methods

Sample selection and histological grading

All samples were collected by the Molecular Mechanisms in Malignant Lymphomas (MMML) network project, for which central and local ethical approval had been obtained. Due to the retrospective nature of the study, patients had been treated with various chemotherapy regimens, including (although only in a few cases) rituximab.

Altogether, 98 tumor samples were included: 12 FL1, 10 FL2, 16 FL3A, 6 FL3B with a purely follicular growth pattern, 9 DLBCL with an additional FL3B component (DLBCL/FL3B), and 45 DLBCL of GCB-type, as determined by gene expression profiling.¹⁰ All tumor samples were classified and graded on the basis of routine hematoxylin and eosin (H&E), Giemsa and Periodic acid Schiff (PAS) stainings according to the criteria of the World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues within a panel review process conducted by expert reference hematopathologists of the MMML.^{1,10}

Immunohistochemical staining, fluorescence *in situ* hybridization and gene expression profiling

Paraffin sections were immunostained with antibodies against CD20, CD10, *BCL2*, *BCL6*, IRF4/MUM1 and Ki67 as previously described.^{10,11} For the detection of *BCL2*, *BCL6*- and

MYC-translocations, FISH was performed as described in the *Online Supplementary Appendix*.¹² Gene expression profiles were generated as described in the *Online Supplementary Appendix*.¹⁰

Statistical analysis

Differential gene expression analysis, ANOVA, determination of gene expression indices, classification analyses, and evaluation of the clinical outcome were assessed as described in the *Online Supplementary Appendix*.

Results

Immunohistochemical and FISH analyses delineate FL1-3A from FL3B and DLBCL/FL3B

According to FISH analyses, *BCL2* breaks were the predominant genetic feature of FL1/2 (18 of 22, 82%). The number of cases with *BCL2* alterations was lower in FL3A (12 of 16, 75%), FL3B (3 of 6, 50%) and in GCB-DLBCL (13 of 43, 30%), and *BCL2* breaks were only infrequently detected in DLBCL/FL3B (1 of 9, 11%) (Table 1 and *Online Supplementary Figure S1A*). The substantial difference in the incidence of *BCL2* breaks between FL3B and DLBCL/FL3B in comparison with FL1/2 or FL3A did not achieve statistical significance due to the small number of cases. Rearrangements of the *BCL6* gene locus were most frequently encountered in DLBCL/FL3B (4 of 9, 44%) and in GCB-DLBCL (15 of 44, 34%), but were also detected in FL1/2 (2 of 22, 9%), FL3A (5 of 16, 31%), and FL3B (1 of 6, 17%) (Table 1 and *Online Supplementary Figure S1A*).

Signal constellations indicative of an *MYC* break were most frequently observed in DLBCL/FL3B (2 of 9, 22%) and, to a lesser extent, also in FL3B (1 of 6, 17%) and GCB-DLBCL (4 of 44, 9%). Occasional *MYC* alterations were also detected in FL1/2 (1 of 22, 5%) and FL3A (1 of 16, 6%) (Table 1 and *Online Supplementary Figure S1A*). According to immunohistochemistry, CD10 positive samples (>25% positive cells) were equally distributed within FL1/2 (13 of 21, 62%), FL3A (8 of 12, 67%), and FL3B/DLBCL (4 of 7, 57%), and, to a lesser extent, in GCB-DLBCL (14 of 42, 33%). CD10 was also expressed in 2 of 2 FL3B tested (Table 1 and *Online Supplementary Figure S1B*). While none of the FL1/2 were IRF4/MUM1 positive ($\geq 26\%$), reactivity for this protein was significantly increased in FL3A (4 of 8, 50%; $P < 0.001$), FL3B (2 of 3, 67%; $P < 0.001$), and DLBCL/FL3B (2 of 6, 33%; $P < 0.05$) (Table 1). With increasing grade, the number of cases with high Ki67 indices ($\geq 70\%$) rose. While 3 of 20 FL1/2 (15%) showed reactivity for Ki67 of 70% or over, such a staining pattern was observed in 4 of 15 FL3A (27%) and in 3 of 5 FL3B (60%; $P < 0.05$). A higher proportion of DLBCL/FL3B (7 of 8, 88%) were Ki67-high, although the difference did not reach significance when compared with FL3B or DLBCL (20 of 39, 51%) (Table 1 and *Online Supplementary Figure S1B*).

Tumor samples with high *BCL2* (>50%) expression were equally distributed among the different subtypes. FL3B and GCB-DLBCL showed the lowest numbers of *BCL2* expressing cases (2 of 4, 50% and 24 of 43, 56%, respectively, vs. a mean frequency of 84% in the other lymphoma subtypes) (Table 1). All samples showed high numbers of *BCL6*-expressing cells ranging from 71% to 100% (Table 1).

To summarize, FISH and immunohistochemical profiles pointed to a profound biological difference between FL1/2

Table 1. Clinical data, immunohistochemistry and FISH analysis of all follicular lymphoma (FL) subsets.

	FL1/2	FL3A	FL3B	FL3B/ DLBCL	GCB- DLBCL	P						
						FL1/2 vs. FL3A	FL1/2 vs. FL3B	FL1/2 vs. DLBCL/FL3B	FL1/2 vs. DLBCL	FL3A vs. FL3B	FL3B vs. DLBCL/FL3B	FL3B vs. DLBCL
N. of cases	22	16	6	9	44							
Median age, years (range)	58 (38-78)	57 (36-71)	53 (39-66)	57 (34-80)	60 (8-85)	ns	ns	ns	ns	ns	ns	ns
Male/female	7/8	5/7	4/0	3/6	24/19	ns	ns	ns	ns	ns	ns	ns
Median OS, months	69	72	55	98	45	ns	ns	ns	ns	ns	ns	ns
<i>BCL2</i> -Break (%)	18/22 (82)	12/16 (75)	3/6 (50)	1/9 (11)	13/43 (30)	ns	ns	0.0007	0.00004	ns	ns	ns
<i>BCL6</i> -Break (%)	2/22 (9)	5/16 (31)	1/6 (17)	4/9 (44)	15/44 (34)	ns	ns	0.023	0.029	ns	ns	ns
<i>MYC</i> -Break (%)	1/22 (5)	1/16 (6)	1/6 (17)	2/9 (22)	4/44 (9)	ns	ns	ns	ns	ns	ns	ns
CD10 (%)	13/21 (62)	8/12 (67)	2/2 (100)	4/7 (57)	14/42 (33)	ns	ns	ns	0.03	ns	ns	ns
IRF4/MUM1 (%)	0/16 (0)	4/8 (50)	2/3 (67)	2/6 (33)	20/39 (51)	0.0009	0.00005	0.014	0.0001	ns	ns	ns
Ki67 (%)	3/20 (15)	4/15 (27)	3/5 (60)	7/8 (88)	29/42 (69)	ns	0.0359	0.00006	0.00003	ns	ns	ns
<i>BCL2</i> (%)	15/20 (75)	12/15 (80)	2/4 (50)	5/7 (71)	24/43 (56)	ns	ns	ns	ns	ns	ns	ns
<i>BCL6</i> (%)	15/17 (88)	12/13 (92)	3/3 (100)	5/7 (71)	34/39 (87)	ns	ns	ns	ns	ns	ns	ns

DLBCL: diffuse large B-cell lymphoma; OS: overall survival; N: number; ns: not significant.

and the other FL-subgroups, with FL3A showing features more similar to FL1/2 than the other subtypes.

Results of gene expression profiling within different FL subtypes by unsupervised analysis

In order to clarify whether FL subtypes defined by cytomorphology do also harbor unique gene expression profiles, hierarchical clustering was performed in 44 FL: FL1 (n=12), FL2 (n=10), FL3A (n=16) and FL3B (n=6). With regard to the 100 most variably expressed genes, however, the different samples did not cluster together according to their pre-defined histological grade (*Online Supplementary Figure S2A and B*). In order to clarify the relationship of tumors with a follicular background, particularly FL3B versus GCB-DLBCL, hierarchical clustering of the 44 FL samples and 45 GCB-DLBCL was performed using two different strategies. 1) The first round of analysis was based on the 100 most variably expressed genes, and 2) the second round focussed on the 500 most variably expressed genes. However, the GCB-DLBCL samples did not cluster together in either of the two approaches, and the 6 FL3B samples were scattered in between FL1-3A and DLBCL (*Online Supplementary Figure S2C and D*).

Gene expression profiling reveals a close relationship between FL3A and FL3B with supervised analysis

In the next step, differential gene expression analysis (DEA) was performed to compare the gene expression profiles (GEP) of tumor subtypes and to align the results to different molecular features. To this end, the differential

gene expression between: i) FL1/2 and FL3A; ii) FL3A and FL3B; iii) FL3B and DLBCL/FL3B; iv) FL3B and DLBCL; v) FL3A and DLBCL; and vi) FL1/2 and DLBCL was assessed. The GEPs of FL1/2, FL3A and FL3B differed significantly from DLBCL (with 7059 probeIDs mapping to 5027 annotated unique genes), 5093 (3798 genes) and 840 (691 genes) differentially expressed, respectively. Intriguingly, comparison of FL1/2 and FL3A revealed significant differences in GEPs (Figure 1A), while both FL3A and FL3B, as well as FL3B and DLBCL/FL3B, showed similar expression patterns (*data not shown*). DEA between FL1 versus FL2 failed to disclose significantly up- or down-regulated genes. Since differential GEPs had been described for t(14;18)-positive and t(14;18)-negative FL, respectively,¹³ the t(14;18) status for each sample was included in the final model to account for potentially hidden confounding effects. This ensures that the final list of genes differentially expressed between FL1/2 and FL3A does indeed reflect differences in regulation between these two FL entities and is therefore not due to the sample's individual t(14;18) status. Comparing FL1/2 and FL3A, 643 differentially expressed genes were identified; of those, 519 genes were up-regulated in FL3A and 125 genes up-regulated in FL1/2. A robust estimate of the median expression of all 643 regulated genes was calculated, resulting in a single value per sample, termed expression index. The FL1/2 subgroup tended to have mainly negative values since the majority of regulated features were indeed down-regulated. In contrast, the samples in the FL3A subtype were mostly positive. Obviously, the

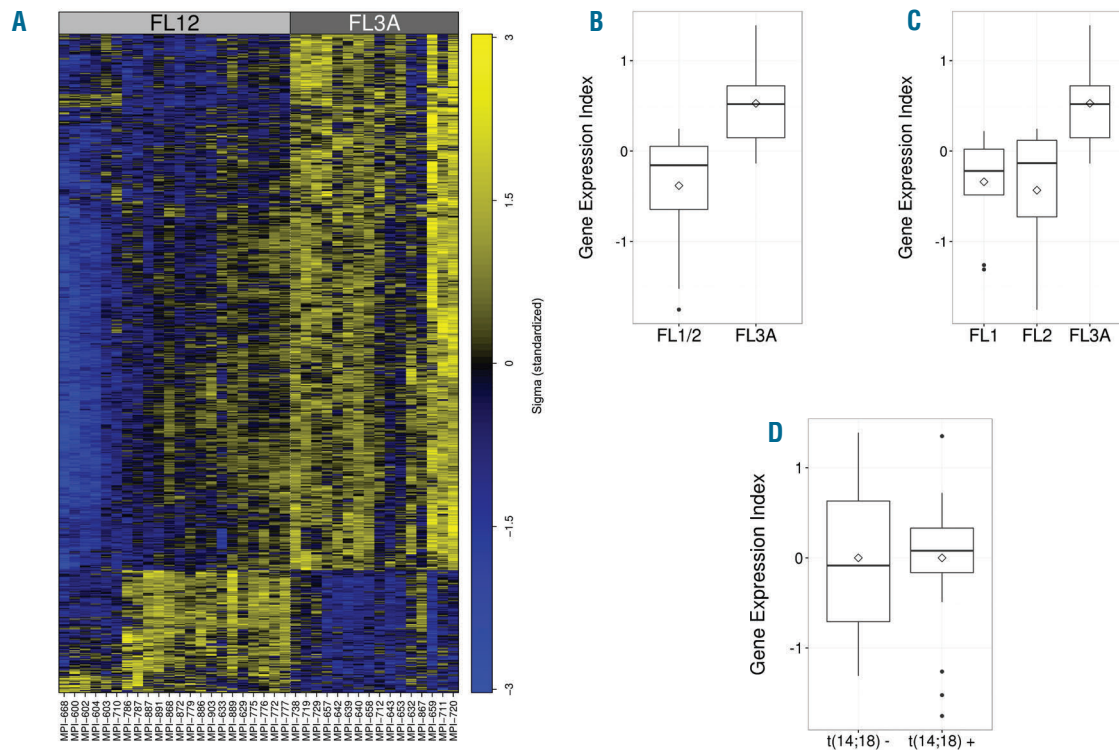


Figure 1. Differential gene expression of FL1/2 and FL3A is independent of t(14;18)-status. Heatmap visualizing all 747 probe IDs differentially expressed between the histological follicular lymphoma (FL) subtypes FL1/2 and FL3A. Gene expression is shown as a pseudocolored representation of log expression ratio (=fold change), with yellow being above and blue being below the median level of gene expression in each row, as shown by the color scale (A). A robust estimate of the median expression of all 747 differentially expressed probe IDs was calculated resulting in a single value per sample (=expression index). While the indices of the FL1/2 subgroup were mainly negative (=down-regulated gene expression), the values for the FL3A group were mostly positive (=up-regulated gene expression). In each boxplot, the diamond symbol represents the mean index value (B). Separating the FL1/2 group into its individual components revealed that the mean indices between FL1 and FL2 are almost identical to FL3A (C). The frequency of the t(14;18) is almost balanced in FL1/2 (75%) and FL3A (82%), and also the mean gene expression indices of t(14;18)-positive and t(14;18)-negative FL are almost identical. This supports the notion that the different expression profiles of FL1/2 and FL3A were not the result of differences in the t(14;18)-status (D).

two means of the two groups were quite distinct, and this had also been supported by the results from DEA (Figure 1B). Splitting up the FL1/2 group into its two components (FL1 and FL2) showed the mean indices of these two groups to be almost identical (Figure 1C).

The list of differentially expressed genes in FL1/2 and FL3A should be independent of the t(14;18)-translocation status, suggesting that the mean gene expression indices of both t(14;18)-positive and t(14;18)-negative samples, should be balanced. Of note, almost identical indices of differentially expressed genes were observed between FL1/2 and FL3A with regard to their t(14;18)-status. Thus, a confounding effect mediated by t(14;18) can be excluded (Figure 1D).

As can be concluded from 519 genes up-regulated in FL3A and 125 genes up-regulated in FL1/2, different signaling pathways are active in the respective subtypes. While FL1/2 is dominated by the expression of genes involved in microenvironmental interactions (as for example cell-cell-adhesion and T-cell proliferation), FL3A is characterized by the expression of genes related to RNA transport and regulation, cell cycle, and DNA repair (Online Supplementary Tables S1 and S2, and Online Supplementary Figures S3 and S4).

Since it might be argued that the differential gene expression observed between FL1/2 and FL3A might only

reflect enhanced proliferation in the latter, previously published proliferation signatures^{14,15} were investigated within the present study cohort. A comparison of genes contained in the known proliferation signature (n=592)¹⁴ with the 643 differentially expressed genes in FL1/2 and FL3A/B showed only a small overlap in 105 genes (approx. 16%). Applying a 'proliferation index' based upon the genes of the published signature¹⁴ to our FL cases revealed a highly heterogeneous spectrum of the indices in FL1/2, indicating 10 of 22 (45%) with low (≤ 0) and 12 of 22 (55%) samples with high proliferation index (> 0) (Online Supplementary Figure S5). Furthermore, differential GEP between FL1/2 and FL3A might reflect the non-malignant stroma, as indicated also by enhanced expression of microenvironmental genes in FL1/2. By analyzing a previously published stromal signature¹⁶ within the present data set, the highest median stroma index was indeed observed in FL1/2 (Online Supplementary Figure S6).

No significant differences were observed when comparing GEPs between FL3A and FL3B. Moreover, upon applying a supervised ANOVA approach including FL1/2, FL3A and FL3B, as previously described,⁸ 12 genes could be identified as significantly differentially expressed between FL1/2 and FL3A (Figure 2A); the majority of these genes are involved in cell proliferation, DNA repair, cellular metabolism and intracellular protein trafficking (Online

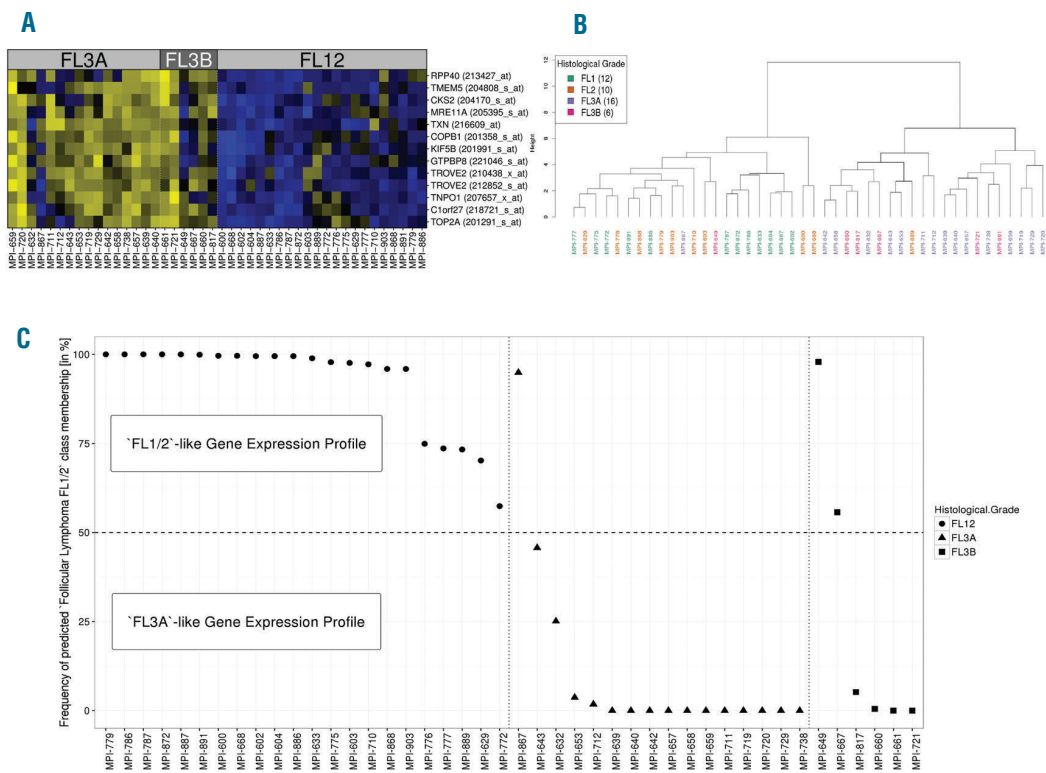


Figure 2. The gene expression profiles of FL3B and FL3A seem to be closely related. A supervised ANOVA analysis identified 13 probe IDs (=12 unique genes) that were most variable between FL1/2, FL3A and FL3B. The majority of FL3B samples more closely resembled the expression pattern of FL3A (A), which was also evident when performing hierarchical clustering based upon the expression of the 13 probe IDs. The legend indicates how many samples belong to an individual entity [eg. FL1(12) represents 12 FL1 samples] (B). Application of a linear classifier that separates FL1/2 from FL3A and subsequently predicts class membership of six FL3B cases revealed that the majority of these FL3B (4 of 6, 67%) show a "FL3A"-like Gene Expression Profile. This result further indicates that the close relationship between FL3A and FL3B is not due to a methodological artefact introduced by ANOVA analysis because the classification approach uses a distinct strategy. The dashed vertical line at Y intercept at 50% separates the 'FL1/2'-like and 'FL3A'-like Gene Expression Profiles. Each follicular lymphoma (FL) entity is represented by an individual symbol (circle: FL1; triangle FL3A; square FL3B). The vertical dotted lines separate the three FL entities (C).

Supplementary Table S3). Interestingly, however, and in contrast to previous findings,⁸ in this analysis, 5 of 6 FL3B samples showed a GEP similar to the FL3A cases (Figure 2A) which was also shown when applying hierarchical clustering to FL subtypes based upon the 12 most differentially expressed genes (Figure 2B).

To further substantiate these findings, supervised classification analysis was performed establishing a linear classifier to separate FL1/2 from FL3A in a test setting and to predict class membership of FL3B in the validation set. The relative frequency (as %) of being classified as FL1/2 (>50%) is indicated in Figure 2C. While 17 of 22 FL1/2 (77%) were clearly classified as such, the GEP of 5 FL1/2 (23%, MPI-629, MPI-772, MPI-776, MPI-777 and MPI-889) was intermediate between FL1/2 and FL3A. With regard to FISH and/or immunohistochemical parameters, there was no difference in the FL1/2 classified as such and the 5 spiking FL1/2, most notably also not in respect of the proliferation index as measured by Ki67 (*data not shown*). Moreover, 13 of 16 FL3A (81%) were explicitly classified as such, while GEP of 2 FL3A (13%, MPI-632 and MPI-643) tended towards the profile of FL1/2. One FL3A (6%, MPI-867) was distinctly classified as FL1/2. All 3 'misclassified' FL3A harbored only low numbers of Ki67-positive cells (mean: 18%, range 5-30%) as the only striking feature when compared with their 'classical' counterparts

(mean: 63%, range 30-90%). Interestingly, categorization of FL3B samples assigned 4 cases (4 of 6, 67%; MPI-660, MPI-661, MPI-721 and MPI-817) as FL3A, while 1 FL3B (17%, MPI-649) was classified as FL1/2, and the expression pattern of the remaining sample (MPI-667) was intermediate between FL1/2 and FL3B. When considering genetic and immunohistochemical features, the 2 'misclassified' FL3B were IRF4/MUM1 expression negative, although a high proportion of FL3B showed positivity for this marker (Table 1). A decreased number of Ki67-positive cells, however, was not observed in these 'misclassified' FL3B samples.

Gene expression profiles of FL3B/DLBCL cluster in between FL3B and GCB-DLBCL

To assess whether FL3B and DLBCL/FL3B show a GEP more similar to FL or DLBCL, classification analysis was undertaken by devising a linear classifier allowing for the distinction of FL1/2 and DLBCL, since GEP clearly separated these entities, with 7059 probe IDs (5027 genes) being differentially expressed. Subsequently, the linear classifier was used to predict class-membership of FL3A, FL3B and FL3B/DLBCL (Figure 3A). GEPs of FL1/2 and DLBCL appeared quite distinct. A homogenous GEP of FL1/2 and DLBCL was observed with only a few exceptions. Of note, 1 DLBCL (MPI-226) was classified as FL1/2 in

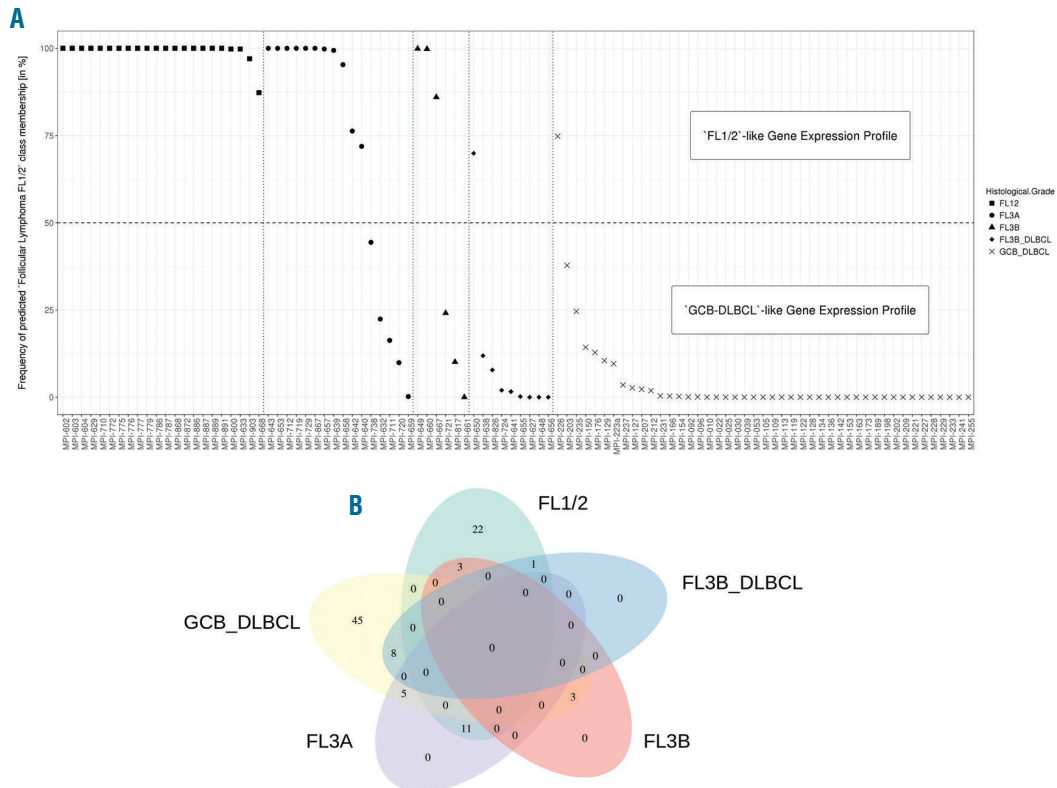


Figure 3. Classification analysis to follicular lymphoma (FL) subtypes FL3A, FL3B and DLBCL/FL3B into either the category of FL1/2 or germinal center B-cell diffuse large B-cell lymphoma (GCB-DLBCL). While the Gene Expression Profiles (GEPs) were clearly separated for FL1/2 and GCB-DLBCL, no clear distinction was seen for FL3A and FL3B. DLBCL/FL3B tended to cluster in the group of GCB-DLBCL (A). Schematic overview of FL subtype categorization based on GEP. While FL1/2 and GCB-DLBCL indicate a homogeneous expression profiling clearly separated from each other, the expression pattern of FL3A and FL3B is in between FL and DLBCL. DLBCL/FL3B are more closely related to DLBCL than to FL (B).

approximately 75% of all iterations. Eleven of 16 (69%) FL3A clustered within the group of FL1/2 (Figure 3A). Considering genetic features of the resulting 5 ‘misclassified’ FL3A, the frequency of *BCL6* breaks in this group (3 of 5, 60%) was more similar to the DLBCL group than to FL1/2 (GCB-DLBCL: 44% vs. FL1/2: 9%) (Table 1). Of interest in this context, the GEP of 3 FL3B (MPI-661, MPI-721 and MPI-817; 3 of 6, 50%) was more closely related to DLBCL than to FL1/2, while the remaining 3 FL3B (50%) were clearly classified as FL1/2 (Figure 3A). However, there was no significant difference in genetic features of the 2 FL3B clusters, especially not with respect to the occurrence of the t(14;18). With special regard to immunohistochemical markers, no differences were detected that could possibly separate the ‘core’ group from the outliers.

The majority of the DLBCL/FL3B samples (8 of 9, 89%) were classified as DLBCL, apart from MPI-650 which was classified as FL1/2 in more than 70% of all iterations (Figure 3A). The only striking finding in this sample was the lack of a *BCL6*-translocation, while 44% of either DLBCL/FL3B or DLBCL, respectively, harbored the rearrangement.

To summarize, gene expression profiling and classification analysis showed FL1/2 to be clearly separated from DLBCL, indicating a distinct FL-specific and DLBCL-specific gene expression pattern, as would have been expected. FL3B turned out to be closely related to FL3A, not categorizing within a separate gene expression cluster, and

both FL3A and FL3B showed overlapping GEPs in between FL1/2 and DLBCL. Finally, based upon their expression pattern, DLBCL/FL3B did indeed seem to represent a composite form of FL3B and DLBCL, with the majority of samples more closely resembling DLBCL (Figure 3B).

Gene expression profiles of FL with or without the t(14;18) do not differ significantly in the various histological subtypes

Presence or absence of the t(14;18) was one of the factors most distinguishing FL1-3A from FL3B, DLBCL/FL3B and GCB-DLBCL (see above). We, therefore, asked whether GEP might be different in lymphomas with or without the t(14;18). Interestingly, such a difference was not seen either in the entire FL cohort, nor within different histological subtypes. Only 2 genes (*FAM30A* and *IL17RB*) were differentially expressed between t(14;18)-positive and t(14;18)-negative FL1/2, FL3A and FL3B (n=33 and n=11, respectively). Upon classification analysis, trying to separate t(14;18)-positive from negative FL1-3B samples, GEP of t(14;18)-negative FL1-3B was quite homogenous, while the classification profiles of some t(14;18)-positive cases fluctuated quite heavily, with 6 FL with t(14;18) (MPI-600, MPI-604, MPI-640, MPI-659, MPI-667 and MPI-668) (6 of 33, 18%) showing a GEP more similar to the t(14;18)-negative cohort (Figure 4A). When testing for the differential expression of single genes that varied between

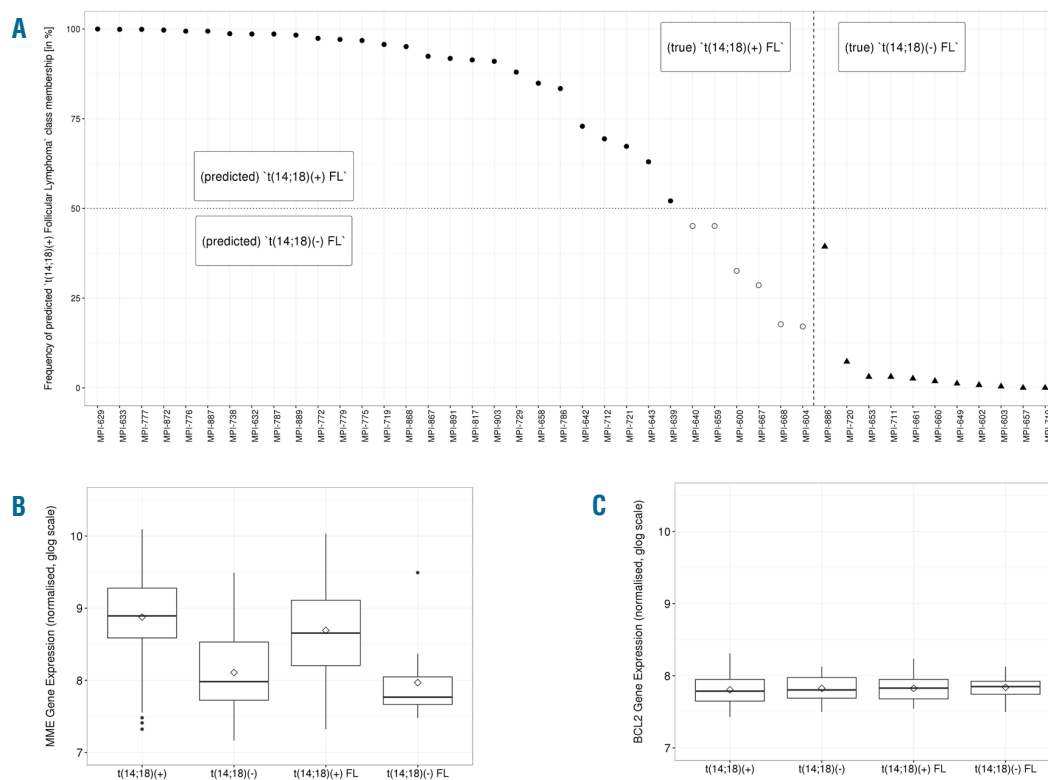


Figure 4. Gene expression profiles and overall survival of follicular lymphoma (FL) with and without t(14;18). Classification analysis of t(14;18)-positive and t(14;18)-negative FL revealed that the expression profile of t(14;18)-negative samples was homogeneous, while the profile of t(14;18)-positive FL fluctuates, with 6 t(14;18)-positive FL that were mainly classified as t(14;18)-negative (A). Gene expression of *CD10* (=MME) was only significantly different when comparing the entire group of t(14;18)-positive and t(14;18)-negative lymphomas [including germinal center B-cell diffuse large B-cell lymphoma (GCB-DLBCL), first two boxplots from the left], but not when comparing FLs only (boxplots 3 and 4). Only a trend towards lowered CD10 expression in t(14;18)-negative FLs was observed. In the boxplot, the mean expression of CD10 is represented by a diamond symbol in addition to its median value (B). Gene expression of *BCL2* showed no significant differences at all, neither in the entire group of t(14;18)-positive and t(14;18)-negative lymphomas, nor in the FL group only (C).

t(14;18)-positive and t(14;18)-negative FL samples in another cohort (e.g. *BCL2*, *BCL6*, *CD10*, *IRF4/MUM1*, *IKBKE*),¹³ a significant difference was detected only for *CD10* (=MME) (Figure 4B), while the median expression of the other genes was almost identical in both groups (e.g. *BCL2* expression in Figure 4C).

Clinical outcome of different histological FL subtypes

In this small series, no significant differences were noted in the overall survival (OS) of FL1/2, FL3A, FL3B and FL3B/DLBCL, which ranged from 55 to 98 months (Table 1 and Figure 5A). In agreement with previous studies,^{13,17} no difference in OS was observed when comparing t(14;18)-positive and t(14;18)-negative FL of all subtypes (FL1/2, FL3A and FL3B) (Figure 5B).

Discussion

The up-dated WHO classification for lymphoid neoplasms categorizes FL into three histological grades according to the number of centroblasts and the presence or absence of centrocytes within the tumor follicles.^{1,18} The vast majority of FL are FL1/2, and hence have formed the backbone of a plethora of reports defining genetic features underlying FL pathogenesis.¹⁹ In contrast, data on grade 3 FL are scarce, obviously due to the limited number of

these cases available for studies. In particular, few studies have been conducted to elucidate the molecular mechanisms of the pathogenesis of FL3B. With respect to available immunophenotypic and genetic data, the two subgroups of FL3 are regarded as discrete entities, with FL3A more closely related to FL1/2, while FL3B, in contrast, to a greater extent resembles DLBCL.^{2,6,8,20,21} This hypothesis is supported by the fact that FL3A frequently harbor FL1/2 follicles in a given tumor specimen and harbor the t(14;18) in roughly 60% of cases, while FL3B only show infrequent *BCL2* translocations, while they are often CD10 negative and IRF4/MUM1 positive.^{2,6,8,20,21}

These differences were also evident in the present study, showing a linear decrease in the frequency of *BCL2* rearrangements (although not significant), as well as an increase in positive stainings for IRF4/MUM1 with the number of centroblasts. A CD10⁻IRF4/MUM1⁺ immunophenotype has been described as typical for FL1/2, while an increased frequency of CD10⁻IRF4/MUM1⁺ cells was reported in FL3B and DLBCL/FL3B.^{6,9} In the present study, a characteristic CD10-negative phenotype was not observed in FL3B, obviously due to the fact that only 2 of the 6 FL3B analyzed had CD10 stainings available. Nevertheless, present data support the concept that FL3A and FL3B are characterized by a different spectrum of underlying genet-

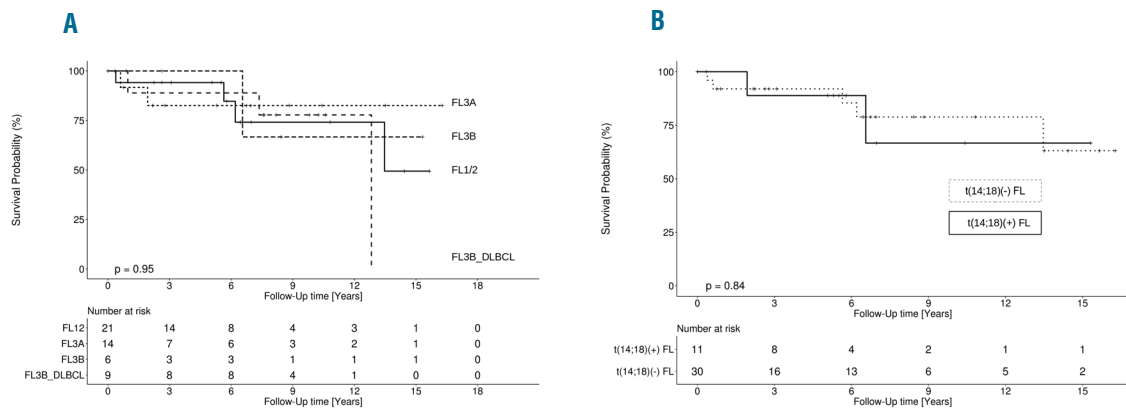


Figure 5. Overall survival (OS) of histological follicular lymphoma (FL) subtypes and FL with and without t(14;18). Kaplan-Meier-plot of the different FL subtypes FL1/2, FL3A, FL3B and diffuse large B-cell lymphoma (DLBCL)/FL3B (A), as well as the comparison of t(14;18)-positive and t(14;18)-negative FL (B) revealed no significant differences in OS.

ic events, suggesting that they are biologically distinct.

In order to obtain a more detailed insight into the pathogenesis of FL3A and FL3B, GEP had been performed by Piccaluga *et al.*⁸ However, they had not integrated markers for immunohistochemistry and FISH into their study. The main finding of this study was a relatively homogeneous GEP of different FL subtypes. In a supervised analysis approach, Piccaluga *et al.*⁸ found that FL1/2 and FL3A formed one cluster, while FL3B formed a separate group distinguishable from FL1/2/3A based on the differential expression of 30 genes. In contrast to these findings, we failed to observe a significant difference in the gene expression patterns of FL1/2 and FL3A on the one hand and of FL3B on the other hand. In contrast, from our data set, a significantly differential gene expression emerged between FL1/2 and FL3A, while FL3B profiles more closely resembled those of FL3A. Despite these different findings concerning the relationship of FL1/2, FL3A and FL3B, we and Piccaluga *et al.* identified similar pathways affected in FL1/2 and FL3, mainly targeting cellular metabolism, cell cycle, and cell growth. Applying previously published proliferation signatures^{14,15} to our FL samples, however, revealed a highly heterogeneous spectrum of proliferation indices in FL1/2 cases in the present study. In keeping with the fact that almost no overlap was observed between the genes from known proliferation signatures and the 643 differentially expressed genes between FL1/2 and FL3A, we provide evidence that proliferation is not the only explanation for the difference between the GEP in FL1/2 and FL3A. An increased proliferation, in particular in FL1/2 samples, had already been described by using miRNA profiling, further substantiating the finding of a wide proliferation spectrum even in FL1/2.^{22,23}

Of 12 genes distinguishing FL1/2 from FL3A/B, all were over-expressed in FL3A/B. Intriguingly, 3 genes, *MRE11A*, *TXN* and *TOP2A*, had already been associated with the pathogenesis of lymphoma and, therefore, targeting their expression might be beneficial for tailored therapy (Online Supplementary Table S3).^{24,26}

Based on the classification approach performed in the present study, FL1/2 were clearly distinguishable from GCB-DLBCL, while FL3A and FL3B, in contrast, showed

highly similar gene expression patterns; moreover, they both formed an expression cluster intermediate between FL and DLBCL. Piccaluga *et al.* found that FL3A and FL3B were clearly distinguishable in their gene expression patterns in supervised analysis and that both subgroups resembled more closely FL than DLBCL.⁸ Nevertheless, in their study, they identified 2 FL3B that clustered within the DLBCL group, similar to the findings in the present study.⁸ These data might indicate that a distinct classification of FL subtypes based on their GEP is not possible in all cases. Furthermore, these results underline the fact that the grouping of FL3B within FL1-3A might not be biologically justified in all cases. In the Kiel classification system,²⁷ FL3B had been regarded as a follicular variant of DLBCL (“follicular centroblastic lymphoma”).

Since so far only two GEP global studies on FL are available, each one analyzing only a limited number of samples, it is difficult to draw universal conclusions from these investigations and, therefore, additional validation studies are clearly needed. In fact, considering only gene expression data, no clear-cut pattern that might be useful to distinguish tumors with a different follicular component can be obtained. Although only a limited number of FL3B were available to be investigated within the present study, the fact that GEP clearly separated FL1/2 and FL3A/FL3B suggests a close relationship between FL3A and FL3B. This notion, however, is in contrast to immunohistochemical and genetic profiles of the different histological FL subtypes that point to a closer relationship between FL1/2 and FL3A, and separating them from FL3B. This phenomenon could possibly be explained by the different methodological approaches used, focusing on the examination of tumor cells by immunohistochemistry and FISH, while both tumor and non-malignant bystander cells are simultaneously interrogated by gene expression profiling.

Finally, the therapeutic implication of a diagnosis of FL3A is still a subject of debate. Many hemato-oncologists regard FL3A as belonging to the spectrum of conventional FL1/2. On the other hand, a recent retrospective analysis of FL3A cases enrolled in the German low- and high-grade lymphoma trials failed to observe any difference in survival between FL3A and FL3B and, most intriguingly,

found a plateau formation in FL3A after six years without late progression-free survival events, in contrast to FL1/2.³ This observation may well relate to the fact that the present study found GEPs to be distinct between FL1/2 and FL3A, and supports a hypothesis of a differential molecular background that might influence the clinical outcome of patients with FL.

Furthermore, recent clinical data showed that patients with DLBCL/FL3B had clinical features comparable to either FL or DLBCL,²⁸ a notion supported by the GEP findings of the present study, pointing to the fact that DLBCL/FL3B may represent a composite form of FL3B and DLBCL with similar gene expression patterns.

Since standardized treatment decisions and distinct therapy strategies for patients with FL still have to be defined, the translation of histological grading into differ-

ent treatment modalities for the moment remains unclear. However, it had previously been postulated that an individualized approach might be warranted in FL treatment since specific molecular signatures have been shown to reflect a clinical aggressiveness of FL that is independent of histological grade.²⁹

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