BCL6-translocations Affect the Phenotype of Follicular Lymphomas Only in the Absence of t(14;18)*IgH/BCL2*

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Abstract. *Background: The translocation t(14;18)IgH/BCL2 is the molecular hallmark of follicular lymphomas (FL). A subset of cases harbours translocations involving the BCL6-gene locus. This study aimed to determine the frequency of BCL2- and BCL6-translocations in FL and to identify morphological and immuno-histochemical features with respect to the presence of BCL2- and BCL6-translocations. Materials and Methods: Fluorescence-in-situ-hybridisation (FISH) was used to determine the BCL2- and BCL6-translocation status of 102 FL and these were compared to morphological and immunohistochemical parameters. Results: Lymphomas with BCL6- and BCL2-translocations were very similar to t(14;18)-positive lymphomas without BCL6-translocations. In contrast, t(14;18)-negative lymphomas with BCL6-translocations were amongst others of higher grade, less often CD10-positive, involved the bone marrow less frequently and did not infiltrate the lymph node capsule. Conclusion: BCL2- and BCL6-translocations correlate with particular phenotypes of follicular lymphomas. BCL6-translocations seem to affect the phenotype only when they are not accompanied by BCL2-translocations.*

Follicular lymphoma (FL) is the second most frequent type of non-Hodgkin's lymphoma (NHL), representing about 20% of NHL in the Western world. The WHO defines FL as a neoplasm of germinal centre cells, which usually has at least a partially follicular growth pattern (1). On the molecular level the translocation t(14;18)*IgH/BCL2* is the hallmark of FL and can be detected in about 80% of cases with varying frequencies depending on the ethnical background and the methods used for the detection of translocations (*e.g.* PCR or fluorescence-in-situ-hybridisation, FISH) (2-14). The juxtaposition of the *BCL2* gene with enhancer sequences of the *IgH* promoter region leads to the constitutive expression of the antiapoptotic bcl2-protein. However, the *BCL2*-translocation alone is not sufficient for malignant transformation of B-lymphocytes (15) and in the majority of FL a multitude of chromosomal aberrations can be found (16-18). Chromosomal translocations involving the *BCL6*-gene locus on 3q27 are seen in about 30% of diffuse large B-cell lymphomas (DLBCL) (19-21). *BCL6* is a transcriptional repressor and has pivotal roles in the formation of germinal centres, lymphocyte differentiation and survival. The frequency of BCL6-translocations in FL is lower (22) with a peak incidence in FL of high histological grade and/or transformation into DLBCL (22-24).

The aim of the present study was to determine the frequencies of *BCL2*- and *BCL6*-translocations in FL and to compare these data to morphological and immunohistochemical parameters in tumour biopsies as well as the bone marrow trephine biopsies.

Materials and Methods

The study was approved by the local ethics committee and included 102 cases of FL. All the cases were primary biopsies before treatment and a staging-trephine biopsy of the bone marrow was available in all cases. The mean age of the patients at diagnosis was 58 yrs. (25-86) with a slight predominance of females (m:f=1:1.2).

All the biopsies were evaluated on H&E-, Giemsa-, Gomori- and PAS-stained slides for the assessment of histological grade according to the WHO-classification (1), growth pattern, sclerosis, marginal zone differentiation and infiltration of the lymph node capsule.

The immunohistochemical reactions were performed on a TechMate®–automatic stainer (Dako, Glostrup, Denmark) using diaminobenzidine as chromogene. The primary antibodies and staining conditions are given in Table I.

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Table I. *Primary antibodies, dilutions and pretreatments.*

Antibody	Company	Clone	Dilution	Pretreatment
CD20	Lab Vision[1]	L26	1:200	steamer (pH 6,1)
CD3	Lab Vision[1]	SP7	1:200	steamer (pH 9,5)
CD4	Lab Vision[1]	4B12	1:50	steamer (pH 9,5)
CD8	Lab Vision[1]	BC/1A5	1:50	steamer (pH 9,5)
CD68	Lab Vision[1]	PGM-1	1:30	steamer (pH 6,1)
CD23	Lab Vision[1]	SP23	1:100	steamer (pH 6,1)
CD10	Menarini[2]	56C6	1:30	steamer (pH 9,5)
Bcl2	Lab Vision[1]	100/D5	1:100	steamer (pH 6,1)
IgD	DAKO[3]	polyclonal	1:50	enzyme
MiB1	DAKO[3]	MIB-1	1:200	steamer (pH 6,1)
Kappa	DAKO[3]	polyclonal	1:20000	steamer (pH 9,5)
Lambda	DAKO[3]	polyclonal	1:20000	steamer (pH 9,5)

[1]Lab Vision, Thermo Fisher Scientific Inc, Fremont, CA, U.S.A.; [2]A. Menarini Diagnostics, Berlin, Deutschland; [3]Dako, Glostrup, Denmark.

The immunohistochemical reactions were evaluated as described previously (25-27). Briefly: CD20 was evaluated as positive or negative. CD3, CD4, CD8 and CD68 were assessed for the percentage of positive cells and their distribution (intrafollicular, perifollicular rimming and diffuse). CD10 and bcl2 were scored as negative and positive (weak and strong); for bcl2, T-lymphocytes were used as internal reference and bcl2-staining was scored as strong if the staining intensity on the B-cells was at least as strong as on the T-cells. CD23 was evaluated with regard to the meshes of follicular dendritic cells (FDC) and the tumour cells; the FDCs were scored as absent, mostly disrupted, partly disrupted and well developed and the tumour cells were scored as negative (<30%), partly positive (30-70%) and positive (>70%). A monotypic light-chain-expression was documented when present or not irrespective of the localization (surface or cytoplasm). IgD was used for the detection of a preserved mantle zone, which was defined as a perifollicular rim of IgD-positive cells encircling at least one third of the follicle. IgD was scored as the percentage of follicles with a preserved mantle zone. MiB1 was scored as the percentage of positive cells with regard to the complete section and the area with the highest proliferation rate ("hot spot").

FISH was performed according to the manufacturers protocol (Abbott Laboratories, Abbott Park, Illinois, USA). Briefly, pepsin and steamer (EDTA, pH 8.0) were used as pretreatments. Probes were hybridized to the sections at 37˚C for 72 hours after denaturation at 80˚C. All the washing steps were performed at room temperature. DAPI was used for counterstaining. The following probes (all purchased from Abbott Laboratories) were used: *BCL2* dual colour break apart rearrangement probe, *IgH/BCL2* dual colour dual fusion translocation probe, *BCL6* dual colour break apart translocation probe. The cut-offs for fusion- and break apart probes were 15% and 10%, respectively, and were established as described previously (28).

Fisher's exact test and Mann-Whitney *U*-test were used for comparison of the morphological and immunohistochemical parameters between different groups of FL. A p-value <0.05 was regarded as significant.

Results

The FISH for the *BCL2*-gene locus was evaluable in 99 cases. Out of these 79 had a t(14;18), one case had a *non-IgH/BCL2*-translocation and one case had a *BCL2*-translocation with equivocal results for the *IgH/BCL2* fusion probe. Three cases lacked a *BCL2*-translocation, but had a gain of the *BCL2*-gene locus. In fifteen cases no structural or numerical aberration could be detected for *BCL2*. Translocations involving the *BCL6*-gene locus were detected in 15/98 cases.

The following analyses focus on the evaluation of FL with and without translocations of the *BCL2*-/*BCL6*-gene loci. Therefore a total of eight cases with equivocal results or numerical aberrations of *BCL2* were excluded. According to the *BCL2*-/*BCL6*-translocation status 94 FL were divided into four groups: i) sixty-nine FL with *BCL2*-translocation lacking BCL6-translocations ($FL^{BCL2+/BCL6-}$), ii) ten FL lacking both *BCL2*- and *BCL6*-translocations ($FL^{BCL2-/BCL6-}$), iii) ten FL with both *BCL2*- and *BCL6*-translocations ($FL^{BCL2+/BCL6+}$), and iv) five FL with *BCL6*-translocation lacking *BCL2*-translocation ($FL^{BCL2-/BCL6+}$). The morphological and immunohistochemical features are summarized in Table II.

$FL^{BCL2+/BCL6-}$. The $FL^{BCL2+/BCL6-}$ represented the most common type of FL. Out of these 57 (83%) were grade 1 or 2 FL, five (7%) grade 3a, six (9%) grade 3b and one (1%) FL with transformation into DLBCL (t-FL). Concerning the morphological features, only a minority of the $FL^{BCL2+/BCL6-}$ (20%) had diffuse areas of at least 25%. Sclerosis was present in 42 (61%), marginal zone differentiation in six (9%), bone marrow infiltration in 35 (51%) and infiltration of the lymph node capsule in 46/53 (87%). The immunohistochemical analyses revealed positivity for CD10 in all and for bcl2 in 67 (97%) $FL^{BCL2+/BCL6-}$, which was strong in most cases (81%). Meshes of FDC were well developed or only partly disrupted in 52 (75%), mostly disrupted in ten (15%) and absent in only seven (10%) of $FL^{BCL2+/BCL6-}$. The tumour cells, however, were mostly negative for CD23 (68%). Preserved follicle mantle zones were detected in 34% and a monotypic light chain expression in 29%. The tumour environment of $FL^{BCL2+/BCL6-}$ consisted amongst others of T-cells (mean 21%), which were dominated by CD4-positive cells (mean CD4/CD8 ratio 2.37). The content of macrophages (CD68) was low and exceeded 10% in only five cases. The distribution of T-cells and macrophages was mostly diffuse. However, in 35 % of cases areas with perifollicular rimming of T-cells were found.

$FL^{BCL2-/BCL6-}$. FL lacking *BCL2*- and *BCL6*-translocations showed some peculiarities. With respect to histological grade, five (50%) were grade 1 or 2 FL, three (30%) grade

Table II. *Comparison of morphological and immunohistochemical features with regard to BCL2- and BCL6-translocations.*

Translocation BCL2/BCL6	pos/neg n=69(%)	neg/neg n=10(%)	*p*-value pos/neg *vs.* neg/neg	neg/pos n=5(%)	*p*-value pos/neg *vs.* neg/pos	pos/pos n=10(%)	*p*-value pos/neg *vs.* pos/pos
Parameter							
grade 1,2/grade 3a,3b,t-FL	57/12	5/5	0.033 *	1/4	0.007*	7/3	n.s.*
grade 1,2,3a/grade 3b, t-FL	62/7	8/2	n.s.*	2/3	0.016*	9/1	n.s.*
diffuse areas ≥25%	14 (20)	7 (70)	0.003*	3 (60)	n.s.*	2 (20)	n.s.*
mean diffuse area (%)	15%	47%	0.009**	40%	n.s.**	19%	n.s.**
capsule infiltration	46/53 (87)	9/9 (100)	n.s.*	0/3 (0)	0.004*	7/9 (78)	n.s.*
BM involvement	35 (51)	3 (30)	n.s.*	0	n.s.*	5 (50)	n.s*
CD10 positive	69 (100)	6 (60)	<0.001*	3 (60)	0.004*	9 (90)	n.s*
bcl-2 positive (strong)	56 (81)	2 (20)	<0.001*	4 (80)	n.s.*	9 (90)	n.s*
CD23 FDC[¶]	52 (75)	6 (60)	n.s.*	2 (40)	n.s.*	5 (50)	n.s.*
C23 tumour cells (>30%)	22 (32)	6 (60)	n.s.*	1 (20)	n.s.*	3 (30)	n.s.*
IgD-pos. mantle zones	23/67 (34)	4 (40)	n.s.*	1 (20)	n.s.*	2 (20)	n.s.*
monotypical light chains	20 (29)	4 (40)	n.s.*	4 (80)	0.036*	6 (60)	n.s*
MiB1, mean (%)[#]	16%	24%	n.s.**	60%	<0.001**	23%	n.s**
MiB1, max. (%)[§]	41%	45%	n.s.**	78%	0.003**	45%	n.s**
$CD3^+$ T-cells (%)	21%	24%	n.s.**	15%	n.s.**	17%	n.s.**
Perifollicular $CD3^+$ T-cells	24 (35)	3 (30)	n.s.*	3 (60)	n.s.*	5 (50)	n.s.*
Intrafollicular $CD3^+$ T-cells	2 (3)	1 (10)	n.s.*	0	n.s.*	0	n.s.*
$CD4^+$ T-cells (%)	15%	19%	n.s.**	10%	n.s.**	15%	n.s.**
Perifollicular $CD4^+$ T-cells	13 (19)	0/9	n.s.*	2 (40)	n.s.*	4 (40)	n.s.*
Intrafollicular $CD4^+$ T-cells	4 (6)	2/9 (22)	n.s.*	0	n.s.*	1 (10)	n.s.*
$CD8^+$ T-cells (%)	8%	9%	n.s.**	6%	n.s.**	9%	n.s.**
Perifollicular $CD8^+$ T-cells	13 (19)	1 (10)	n.s.*	1 (20)	n.s.*	1 (10)	n.s.*
Intrafollicular $CD8^+$ T-cells	1 (1)	0	n.s.*	1 (20)	n.s.*	0	n.s.*
CD4/CD8-Ratio	2,4	2,3	n.s.**	1,7	n.s.**	2,3	n.s.**
$CD68^+$ macrophages >10%	5 (7)	3 (30)	n.s.*	0	n.s.*	2 (20)	n.s.*

BM= bone marrow, [¶]cases with well developed or partly disrupted meshes of follicular dendritic cells, [#]percentage of positive cells with regard to the complete section, [§]percentage of positive cells with regard to the area with the highest proliferation, n.s.=not significant ($p>0.05$), *Fisher's exact test, ***U*-Test.

3a FL, one (10%) grade 3b, and one (10%) t-FL. In comparison to $FL^{BCL2+/BCL6-}$ a diffuse growth pattern (≥25%) was significantly more frequent (70% *vs.* 20%, $p=0.003$) and the $FL^{BCL2-/BCL6-}$ were CD10-positive in only 60% ($p<0.001$). Interestingly, the bcl2-expression was not significantly different with regard to positive and negative cases. However, in contrast to $FL^{BCL2+/BCL6-}$, the bcl2-reactivity was only rarely strong in the $FL^{BCL2-/BCL6-}$ (20% *vs.* 81%, $p<0.001$). The tumour environment was very similar in both groups. There was a slightly higher number of macrophages in the $FL^{BCL2-/BCL6-}$, but the difference lacked significance ($p=0.061$).

$FL^{BCL2-/BCL6+}$. The most obvious differences from the group of $FL^{BCL2+/BCL6-}$ were found in cases with *BCL6*-translocations lacking *BCL2*-translocations. These were more often of higher histological grade with the majority being grade 3 FL and t-FL (80% *vs.* 17%, $p=0.007$), did not infiltrate the lymph node capsule (0% *vs.* 87%, p=0.004) and did not involve the bone marrow (0% *vs.* 51%, $p=0.056$). With respect to the immunophenotype, the higher histological grade was reflected in a significantly higher MiB1-count (mean 60% *vs.* 16%, $p<0.001$). The $FL^{BCL2-/BCL6+}$ were less often CD10-positive (60% *vs.* 100%, $p=0.004$) but more frequently showed monotypic light chain expression (80% *vs.* 29%, $p=0.036$).

$FL^{BCL2+/BCL6+}$. Cases harbouring both, *BCL2*- and *BCL6-translocations*, were very similar to the group of $FL^{BCL2+/BCL6-}$ and no significant differences were found regarding all the tested parameters.

Tumour environment. The microenvironment was assessed by the content and distribution of T-cells (CD3), T-cell subsets (CD4 and CD8) and macrophages (CD68) as well as by the pattern of FDC networks. Neither the content of T-

cells and macrophages nor their distribution differed significantly between the four groups of FL. The pattern of FDC correlated to the growth patterns of the tumours. The FL with a predominant follicular growth patterns showed well preserved FDC whereas cases with diffuse areas showed disrupted FDC networks. However, when the four groups of FL were compared to each other, the differences were not significant.

Discussion

The FL lacking the BCL2-translocation did show morphological and immunophenotypical pecularities. Most studies (10, 22, 29-33), but not all (34), showed that FL^{BCL2-} are more frequently negative for CD10. The present data confirmed that the $FL^{BCL2-/BCL6-}$ were significantly more often CD10 negative than the $FL^{BCL2+/BCL6-}$ ($p<0.001$). This finding may reflect data from a recent study by E. Leich and colleagues (35), who showed that at the mRNA-level FL^{BCL2-} exhibited enrichment for activated B-cell like, NFkappaB, proliferation and bystander cell signatures whereas germinal centre B-cell associated signatures were enriched in FL^{BCL2+}.

Since the translocation t(14;18) leads to constitutive expression of bcl2, a strong correlation between the translocation and immunohistochemical detection of bcl2 would be expected, however, the reports are inconsistent (8, 10, 29, 30, 33, 36). These discrepancies could be explained by different scoring methods and inter-observer variability (26). A three-tired scoring-system (negative, weak and strong) as described by the *Lunenburg Lymphoma Biomarker Consortium* (26) with T-cells as internal reference was used in the present study. Interestingly, the $FL^{BCL2+/BCL6-}$ showed strong reactivity for bcl2 significantly more often than the $FL^{BCL2-/BCL6-}$ ($p<0.001$). But when bcl2-positive cases (weak and strong) were compared to negative cases, the difference lacked significance ($p=0.076$). Thus, not only the mere presence, but also the intensity of expression of bcl2-protein seem to be biologically important.

Diffuse areas were significantly more often present in the $FL^{BCL2-/BCL6-}$ than in the $FL^{BCL2+/BCL6-}$. This held true for the proportion of cases with a diffuse pattern ≥25% ($p=0.003$) as well as for the mean diffuse area (U-test $p=0.009$). In this context Karube and colleagues reported a subtype of CD10-negative MUM1-positive follicular lymphoma that frequently lacked BCL2-translocations and showed diffuse areas (32). The expression of MUM1 was not studied in the present series, but it seems likely that at least a part of the $FL^{BCL2-/BCL6-}$ would have fallen into this category. In addition Katzenberger *et al*. (37) described a peculiar group of FL with a predominant diffuse growth pattern, lack of BCL2-translocation and a deletion in the chromosomal region 1p36. These cases typically presented in the inguinal region and lacked bone marrow infiltration. In the present series only three $FL^{BCL2-/BCL6-}$ had a predominant diffuse growth pattern (focally follicular according to the WHO 2008). None of these had bone marrow infiltration, but only one case was an inguinal lymph node biopsy. Since comparative genomic hybridization (CGH) data were not available, the frequency of del1p36 cannot be commented on in this study.

Previous studies have shown that FL^{BCL2+} presented in younger ages than FL^{BCL2-} (4, 9), but no difference was found regarding the age at diagnosis between $FL^{BCL2-/BCL6-}$ and $FL^{BCL2+/BCL6-}$ in the present study (data not shown).

A *BCL6*-translocation was found in 15% of the FL and 10% had a *BCL6*- and *BCL2*-translocation. These frequencies were in accordance with previous studies: Keller and colleagues reported an incidence of 11% (38) and Diaz-Alderete and colleagues of 9% (36) for $FL^{BCL2+/BCL6+}$. Both studies showed, in accordance with the present data, that $FL^{BCL2+/BCL6+}$ were predominantly low grade FL. No morphological or immunohistochemical feature that was significantly different in $FL^{BCL2+/BCL6+}$ and $FL^{BCL2+/BCL6-}$ could be identified, hence, this study confirmed former findings that $FL^{BCL2+/BCL6+}$ are very similar to $FL^{BCL2+/BCL6-}$ (38, 39).

By contrast, *BCL6*-translocations have a tremendous impact on the phenotype of t(14;18)-negative FL. On the one hand, this study confirmed previous reports that these tumours were typically high grade (23, 24, 36) and therefore had increased proliferation (MiB-1) in comparison to $FL^{BCL2+/BCL6-}$ ($p<0.001$). On the other hand the $FL^{BCL2-/BCL6+}$ did not infiltrate the lymph node capsule ($p=0.004$), a feature that is otherwise rather typical in FL. However, only three $FL^{BCL2-/BCL6+}$ samples included sufficient lymph node capsule for histological evaluation. Therefore, this finding needs to be confirmed in further studies. The finding of decreased CD10-reactivity and an increased detection rate of monotypical light chains in $FL^{BCL2-/BCL6+}$ may be associated with the higher morphological grade and has been described for grade 3 FL and t-FL by Ott *et al*. (24). From a clinical point of view, Diaz-Alderete *et al*. (36) and Jardin *et al*. (31) did not find significant differences with regard to overall survival for $FL^{BCL2-/BCL6+}$ in comparison to $FL^{BCL2+/BCL6-}$. However, a lower rate of bone marrow infiltration has been reported (36, 40) and in the present series there was a trend for a lower incidence of bone marrow infiltration in the $FL^{BCL2-/BCL6+}$ than in the $FL^{BCL2+/BCL6-}$ ($p=0.056$).

Since gene expression experiments (41-43) provided evidence for the prognostic relevance of the tumour microenvironment, several studies, in part with contradictory results, showed that the microenvironment may influence the clinical course of the disease (44-50). Surprisingly, no significant difference in terms of the content and distribution of T-cells, T-cell subsets (CD4 and CD8) and macrophages as well as the development of FDC-networks between the FL with different *BCL2*- and *BCL6*-translocation status were identified

in the present study. However, there was a trend for a higher content of macrophages in the $FL^{BCL2-/BCL6-}$ than in the $FL^{BCL2+/BCL6+}$ (p=0.061). This could correspond to the enrichment of bystander cell signatures in FL^{BCL2-} reported by Leich *et al*. (35).

Overall it seems that FL may be subdivided into three groups: i) t(14;18)-positive FL with or without BCL6-translocation, ii) t(14;18)-negative FL without BCL6-translocation, and iii) t(14;18)-negative FL with BCL6-translocation. Whether these groups have any prognostic implications remains to be elucidated. Since the micromilieu was not found to be affected by *BCL2*- and *BCL6*-translocations, it may be that on a clinical basis, the putatively different biology of the proposed subtypes is overcome by different host responses.

In conclusion, *BCL2*- and *BCL6*-translocations do have an impact on the morphology and immunophenotype of follicular lymphomas. However, our data indicate that *BCL6*-translocations affect the phenotype of FL only if they are not accompanied by *BCL2*-translocations.

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